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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 0531 for a patent by AMRAD OPERATIONS PTY LTD filed on 26 November 1997.

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MANAGER EXAMINATION SUPPORT AND SALES

Regulation 3.2

AMRAD Operations Pty Ltd

A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Compositions"

The invention is described in the following statement:

COMPOSITIONS

FIELD OF THE INVENTION

The present invention relates generally to compositions and more particularly to compositions comprising leukaemia inhibitory factor, (hereinafter referred to as "LIF"). The compositions of the present invention are particularly useful as prolonged shelf-life compositions, compositions which exhibit enhanced stability and/or compositions exhibiting reduced aggregation and/or reduced deamidation of active ingredients .

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BACKGROUND OF THE INVENTION

LIF is a polyfunctional glycoprotein with diverse actions on a broad range of tissue and cell types, including induction of differentiation in a number of myeloid leukaemic cell lines, suppression of differentiation in normal embryonic stem cells, stimulation of proliferation of osteoblasts and DA-1 haemopoietic cells and potentiation of the of the proliferative action of interleukin-3 (IL-3) on megakaryocyte precursors. Functionally, LIF is able to switch autonomic nerve signalling from adrenergic to cholinergic mode, stimulate calcium release from bones, stimulate the production of acute phase proteins by hepatocytes and induce loss of fat deposits by inhibiting lipoprotein lipase-mediated lipid transport into adipocytes.

With a potentially broad range of clinical applications, it is imperative that compositions containing LIF are presented in a stable form and remain so during an extended period which may include shipment, handling and storage. Thus, a stable composition is one which retains its physical, chemical, therapeutic and toxicological profile over this period.

Deamidation is the most significant chemical degradation of LIF over time. It is clearly desirable that this process is minimized. Physical degradation, such as aggregation or flocculation, may occur due to denaturation caused by elevated temperatures and/or agitation and excessive handling of the composition. Such degradation is clearly undesirable in terms of appearance and more importantly, consistent and effective administration of LIF in clinical applications. Storage at temperatures below room temperature typically retards chemical degradation, with storage in the frozen state being generally the most effective. Whilst this may minimize chemical degradation, the process of thawing the composition may then result in aggregation.

Thus, there exists a need for a stable composition and, in particular, a stable pharmaceutical composition of LIF wherein chemical and physical degradation is minimised.

SUMMARY OF THE INVENTION

Accordingly, one aspect of the present invention contemplates a composition with improved chemical and physical stability comprising LIF and suitable stabilizing agents, and one or more pharmaceutically acceptable carriers or diluents.

A further aspect of the invention provides a composition with improved chemical and physical stability comprising LIF and suitable stabilizing agents, and one or more pharmaceutically acceptable carriers or diluents under conditions in which aggregation of LIF is reduced.

Yet a further aspect of the invention provides a composition with improved chemical and physical stability comprising LIF and suitable stabilizing agents, and one or more pharmaceutically acceptable carriers or diluents under conditions in which deamidation of LIF is reduced.

In another aspect the present invention is directed to a stable composition comprising LIF together with one or more pharmaceutically acceptable carriers or diluents, wherein the composition has a pH of between 3.5 - 6.5.

A further aspect the present invention provides a stable composition comprising LIF together with one or more pharmaceutically acceptable carriers or diluents, wherein the composition has a pH of between 3.5 - 6.5 under conditions in which aggregation of LIF is reduced.

A further aspect the present invention contemplates a stable composition comprising LIF together with one or more pharmaceutically acceptable carriers or diluents, wherein the composition has a pH of between 3.5 - 6.5 under conditions in which deamidation of LIF is reduced.

In yet a further aspect of the present invention there is provided a method for preparing a composition with improved chemical and physical stability comprising admixing LIF and suitable stabilizing agents, together with one or more pharmaceutically acceptable carriers or diluents .

Another aspect of the present invention, contemplates a method for preparing a composition with improved chemical and physical stability comprising admixing LIF and suitable stabilizing agents, together with one or more pharmaceutically acceptable carriers or diluents under conditions in which aggregation of LIF is reduced.

Yet a further aspect of the invention is directed to a method for preparing a composition with improved chemical and physical stability comprising admixing LIF and suitable stabilizing agents, and one or more pharmaceutically acceptable carriers or diluents under conditions in which deamidation of LIF is reduced.

In another aspect, the present invention is directed to a method of preparing a stable composition comprising admixing LIF together with one or more pharmaceutically acceptable carriers or diluents, wherein the composition has a pH of between 3.5 - 6.5.

A further aspect the present invention contemplates a method for preparing a stable composition comprising admixing LIF together with one or more pharmaceutically acceptable carriers or diluents, and wherein the composition has a pH of between 3.5 - 6.5, under conditions in which aggregation of LIF is reduced.

In another aspect the present invention provides a method for preparing a stable composition comprising LIF together with one or more pharmaceutically acceptable carriers or diluents, and wherein the composition has a pH of between 3.5 - 6.5, under conditions in which deamidation of LIF is reduced.

Preferred compositions are referred to as pharmaceutical compositions and indicate that LIF is present in a pharmaceutically acceptable composition.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1 to 3 respectively diagrammatically depict representative Reversed Phase, Ion Exchange and Size Exclusion chromatograms for a 1.0 mg/ml standard solution of LIF prepared as described in Example 1 by diluting "stock" solution with 2 mM phosphate buffer, pH 6.42, containing 0.01% polysorbate.

Figure 4 graphically represents LIF concentration for samples at each pH after freeze/thaw cycling.

Figure 5 graphically represents the average concentration over 5 freeze/thaw cycles for each pH value.

Figures 6 to 15 diagrammatically represent IEC chromatograms for samples stored in each of the different buffer systems at 8 and 25°C.

Figures 16 to 18 graphically represent IEC results for samples stored at -80, -20, 8 and 25°C with the main LIF peak plotted as a percentage of the total area for all LIF related peaks in the chromatogram as a function of storage time.

Figures 19 to 21 graphically represent RP results for samples stored at -80, -20, 8 and 25°C with the measure concentration of LIF plotted as a function of storage time.

Figures 22 to 24 graphically represent the SEC results for samples stored at -80, -20, 8 and 25°C with the measured concentration of LIF plotted as a function of storage time.

Figures 25 to 28 diagrammatically represent IEC chromatograms for samples at pH 5.5 stored at 8 and 25°C.

Figures 29 and 30 diagrammatically represent a comparison of chromatograms for samples prepared at pH 5.0 and pH 5.5 stored at 8°C and 25°C for 8 weeks.

Figure 31 graphically represents the stability of LIF at pH 5.5 in citrate buffer.

Figures 32 and 33 graphically represent the IEC stability data for 0.4 mg/ml concentrations of LIF at pH 5.0 and pH 5.5.

Figures 34 to 37 graphically represent the concentration of LIF at pH 5.5 as a function of time as analysed by RP, SEC and IEC assays.

Figures 38 to 39 graphically represent the stability of 0.4 mg/ml LIF compositions containing the stabilizing agents Sorbitol, Tween and NaCl.

Figures 40 and 41 graphically represent SEC data for 0.05 and 0.4 mg/ml formulations of LIF containing the stabilization agents Sorbitol, Tween® 80 and NaCl.

Figures 42 to 44 graphically represent freeze/thaw cycling data for pH 5 citrate buffers containing sorbitol or NaCl as analysed by SEC.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions comprising LIF. The present invention particularly provides LIF in a stable form.

Unless otherwise specified, use of "LIF" herein refers to synthetic recombinant or purified naturally occurring LIF from animal or avian species. Preferred animal species are mammals such as humans, primates, and livestock (e.g. sheep, pigs, cows, goats, donkeys and horses), laboratory animals (e.g. murines species, guinea pigs, rabbits and hamsters), companion animals (e.g. dogs and cats) or captive wild animals (e.g. kangaroos, foxes, and deer). Preferred avian species include but are not limited to caged birds, chickens, ducks and geese. As referred hereinto LIF includes reference to derivatives, homologues and analogues of LIF. Derivatives, homologues and analogues are parts, fragments or portions of LIF which are functionally active. Such derivatives or parts thereof include any one or more contiguous series of amino acids contained within any one of the above LIF molecules and includes single or multiple amino acids substitutions, deletions and/or additions to or in the natural or synthetic LIF molecule as well as hyperglycosolated and deglycosolated forms. Conditions for preparing recombinant LIF are disclosed in International Patent Application Nos PCT/AU88/00093 and PCT/AU90/00001 although these conditions may vary depending on the host cell used. Any such variations and/or modifications are within the scope of the subject invention. The host cells may be eukaryotic (eg. yeast, mammalian, insect, plant etc) or prokaryotic (eg. Escherichia coli, Bacillus sp, Pseudomonas sp etc) cells.

The compositions of the present invention achieve their stability through judicious choice of

pH conditions within the range of between 3.5 - 6.5 and optionally the presence of suitable stabilizing agents. Preferably, the pH range is from about 4.0 - 6.0, more preferably from about 4.5 - 5.5. Most preferably, the pH of the composition is about 5.0.

Suitable stabilizing agents are known to those skilled in the art and include isotonicity agents, agents to increase or maintain the conformational stability of LIF and surfactants. It is understood that one agent may possess more than one stabilizing property and more than one agent may be employed to achieve a stabilizing effect.

Suitable isotonicity agents are those which maintain approximately the same osmotic pressure as that of cellular fluids, and are known to those skilled in the art. These may include, but are not limited to, polyhydric alcohols such as sorbitol, pharmaceutically acceptable salts such as NaCl, buffer species, sugars and pharmaceutically acceptable polymeric compounds . Suitable surfactants may be anionic, cationic, amphoteric or non-ionic. Preferred surfactants include fatty alcohols such as lauryl, cetyl and stearyl alcohols, glyceryl esters such as the mono-, di- and triglycerides, fatty acid esters of fatty alcohols and other alcohols such as propylene glycol, polyethylene glycol, sorbitol, sucrose and cholesterol. Other suitable agents include the polysorbates such as polysorbates 20, 40, 60 and 80 and sorbitan ester, polyoxyethylene derivatives and pharmaceutically acceptable polyoxyethylene-polyoxypropylene copolymers. Suitable agents which maintain or increase the conformational stability of LIF are also known to the person skilled in the art and include sugars and polyhydric alcohols.

Suitable buffers for attaining the desired pH of the composition will be known to those skilled in the art and include phosphate, citrate and acetate buffers. Preferred buffers are citrate and acetate.

The compositions of the present invention may be suitable for administration in a variety of forms such as, but not limited to, parenteral (e.g. intravenous, intraperitoneal, intramuscular, intradermal), subcutaneous, nasal, rectal, vaginal, topical, buccal and sublingual.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as a solution an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

Compositions suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

Compositions for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter.

Compositions suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

Compositions suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening

agents. The compositions may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage compositions are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the active ingredient.

It is also understood that the compositions of the present invention may also comprise one or more active agents such a cytokines e.g. interleukins, CD antigens, colony stimulating factors, interferons, and tissue necrosis factor.

It should be understood that in addition to the active ingredients particularly mentioned above, the compositions of this invention may include other agents conventional in the art having regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

A number of formulations of LIF were investigated in order to establish optimum conditions

under which chemical and physical degradation is reduced compared to the currently employed formulation of 3.67 mg/ml in 2 mM phosphate buffer, pH 6.4-6.8.

Ion Exchange (IE), Reversed Phase (RP) and Size Exclusion (SEC) chromatography were used to detect changes in chemical and physical degradation.

Freeze/thaw studies revealed high solubility of LIF, i.e. no aggregation, in formulations in the pH range of 4.0 - 6.0 examined, the highest being in the pH range of 4.5 to 5.5, with optimized stability at pH 5.0.

Studies of the various solutions over varying periods of storage time (0 to 8 weeks) and at a range of storage temperatures (-80 to 25°C) revealed optimum stability of the solution was achieved in a preferred pH range of 4.5 to 5.5.

The inventors examined a number of pH levels and stabilizing agents. Samples at pH 4.0, 4.5, 5.0, 5.5 and 6.0 were prepared in Examples 1 and 2, as described hereinafter, and additional stabilizing agents, Sorbitol, an isotonicity agent, and Polysorbate 80, as a non-ionic surfactant to reduce non-specific adsorption onto surfaces, including glass, were also included. NaCl was also examined as an isotonicity agent.

LIF is present in the compositions of the invention in effective amounts. Effective amounts include from 0.1 μg/ml to 100 mg/ml. Preferred effective amounts are from 10 μg/ml to 10 mg/ml. Particularly preferred amounts range from 400 μg/ml to 1000 μg/ml.

Suitable amounts of surfactant and isotonic agents may range from 0.001 to 30%. Preferably from 0.01 to 10%, even more preferably from 0.01 to 5.0%.

Particularly preferred compositions are those comprising LIF, sorbitol, polysorbate and a citrate or acetate buffer in the preferred ranges described above.

The invention will now be described with reference to the following non-limiting Examples.

Example 1.

I. Preliminary Formulation Screening

On the basis of preliminary stability data, it was anticipated that deamidation of LIF would represent the principal pathway for degradation of solutions at neutral to slightly alkaline pH. Solution pH was therefore considered to be critical and was the primary variable evaluated in these stability studies. Screening studies evaluating LIF stability during freeze/thaw cycling, following filtration, upon contact with vials and syringes and following temperature controlled storage were conducted in the pH range of 4 to 6 using acetate and citrate buffers at low concentrations (10 mM for each). Osmolality was controlled by the addition of sorbitol at a concentration of 5% w/v. To minimise the potential for LIF adsorption to vials, filters, and syringes, 0.01% w/v Polysorbate 80 was added to all preliminary formulations evaluated in this series of studies.

II. Analytical Methods

Three analytical methods were used to assess LIF stability upon storage. A reversed phase assay, using a standard wide pore C8 reversed phase column, was utilised for the purpose of total LIF concentration determination. The reversed phase assay was not stability indicating and therefore was not suitable for the determination of degradation products. A cation ion exchange assay was used to assess degradation products resulting from a change in the charge characteristics of the parent compound as deamidation had previously been determined to be the principal pathway for LIF degradation. A size exclusion assay was also used to detect size related changes (either cleavage, crosslinking, or aggregation) upon storage.

A. Reversed Phase (RP) Assay

Reversed phase chromatography was conducted using a wide pore C8 reversed phase column, and a trifluoroacetic acid /acetonitrile mobile phase with gradient elution. Detection was conducted at 210 nm.

B. Ion-Exchange (IEC) Assay

Ion exchange chromatography was conducted using a cation exchange column, pH 7 phosphate buffer and a salt gradient. Detection was conducted at 280 nm.

C. Size Exclusion (SEC) Assay

Size exclusion chromatography was conducted using a dextrose based size exclusion column with a molecular weight range of 10 to 300 Daltons. The mobile phase was a pH 7.2 phosphate buffer and detection was conducted at 210 nm.

III. Method Validation

A. Reversed Phase (RP) Assay

Using the defined RP conditions, LIF eluted as a sharp, symmetrical peak with a retention time of approximately 37 min as shown in Figure 1. The RP assay was used for quantitation of total LIF only as the method was not selective for LIF in the presence of degradation (deamidation or dimeric) products.

Calibration curves for total peak area versus LIF concentration were prepared with each set of analyses in the concentration range of 0.2 and 1.0 mg/ml LIF.

Precision was determined from the coefficient of variation (CV, %) for the total peak area obtained for replicate injections of standard solutions prepared at 0.4 and 1.0 mg/ml. Accuracy was determined by comparison of the total peak area for these standard solutions to a separately prepared calibration curve and was expressed as the percentage deviation from the nominal concentration. Results for accuracy and precision with the RP assay are shown in Table 1. A summary of the RP calibration curves is shown in Table 2.

B. Ion-Exchange (IEC) Assay

Using the defined IEC conditions, LIF eluted as a slightly tailing peak with a retention time of approximately 13 min as shown in Figure 2. Separation of the main LIF peak from degradation (deamidation) products formed following storage is shown in chromatograms in

Figures 6 to 15. The actual identity of the degradation products (i.e. site of deamidation) was not determined in these studies.

Calibration curves for total peak area (main peak plus degradation products) versus LIF concentration were prepared with each set of analyses in the concentration range of 0.2 and 1.0 mg/ml LIF. Calibration curves were linear in this range when 100 μl was injected onto the column.

Precision was determined from the coefficient of variation (CV, %) for the total peak area obtained for replicate injections of standard solutions prepared at 0.4 and 1.0 mg/ml. Accuracy was determined by comparison of the total peak area for these standard solutions to a separately prepared calibration curve and was expressed as the percentage deviation from the nominal concentration. Results for precision and accuracy for the IEC assay are shown in Table 3. A summary of the IEC calibration curves over the course of the study is shown in Table 4.

C. Size Exclusion (SEC) Assay

Using the defined SEC conditions, LIF eluted as a sharp, symmetrical peak with a retention time of approximately 26 min as shown in Figure 3. The method separated monomeric LIF from dimeric LIF which eluted at approximately 21 min, but was not selective for other degradation (deamidation) products which eluted as monomeric LIF.

Calibration curves for total peak area (main peak plus degradation products) versus LIF concentration were prepared with each set of analyses in the concentration range of 0.2 and 1.0 mg/ml LIF.

Precision was determined from the coefficient of variation (CV, %) for the total peak area obtained for replicate injections of standard solutions prepared at 0.4 and 1.0 mg/ml. Accuracy was determined by comparison of the total peak area for these standard solutions to a separately prepared calibration curve and was expressed as the percentage deviation from

the nominal concentration. Results for precision and accuracy for the SEC assay are shown in Table 5. A summary of the SEC calibration curves is shown in Table 6.

IV. Buffer Composition

All LIF samples were prepared by dilution of stock LIF solution containing 3.67 mg/ml LIF in 2 mM phosphate buffer, pH 6.42 to give the desired final LIF concentration (either 0.4 or 1.0 mg/ml) and composition of buffer components. In these studies, the final composition of each solution contained 10 mM buffer (either acetate or citrate), 5% w/v sorbitol and 0.01% w/v Polysorbate 80. Samples differed in the final concentration of phosphate buffer (present from the original stock LIF solution) depending on the dilution factor. The 0.4 mg/ml LIF solutions contained 0.22 mM residual phosphate while the 1.0 mg/ml LIF solutions contained 0.54 mM residual phosphate. The composition of each buffer was as follows:

A. Acetate Buffer for 0.4 mg/ml LIF Formulations

Solution A: 11.22 mM sodium acetate trihydrate (Merck #1.06267)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 11.22 mM glacial acetic acid (Sigma Chemicals #A6283)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solutions A and B were mixed to give a final pH of 4.0 or 4.5. Formulations were prepared by combining 0.109 parts stock LIF solution and 0.891 parts buffer to give a final LIF concentration of 0.4 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v.

B. Acetate Buffer for 1.0 mg/ml LIF Formulations

Solution A: 13.75 mM sodium acetate trihydrate (Merck #1.06267)
6.88% w/v sorbitol (Sigma Chemicals #S1876)

0.0138% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 13.75 mM glacial acetic acid (Sigma Chemicals #A6283)
6.88% w/v sorbitol (Sigma Chemicals #S1876)
0.0138% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solutions A and B were mixed to give a final pH of 4.0 or 4.5. Formulations were prepared by combining 0.272 parts stock LIF solution and 0.728 parts buffer to give a final LIF concentration of 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v.

C. Citrate Buffer for 0.4 mg/ml LIF Formulations

Solution A: 11.22 mM sodium citrate dihydrate (Merck #1.06448)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 11.22 mM citric acid monohydrate (Merck #1.00244)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% Polysorbate 80 (Sigma Chemicals #P1754)

Solutions A and B were mixed to give a final pH of 5.0, 5.5, or 6.0. Formulations were prepared by combining 0.109 parts stock LIF solution and 0.891 parts buffer to give a final LIF concentration of 0.4 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v.

D. Citrate Buffer for 1.0 mg/ml LIF Formulations

Solution A: 13.75 mM sodium citrate (Merck #1.06448)
6.88% w/v sorbitol (Sigma Chemicals #S1876)
0.0138% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 13.75 mM citric acid (Merck #1.00244)
6.88% w/v sorbitol (Sigma Chemicals S1876)
0.0138% w/v Polysorbate 80 (Sigma Chemicals P1754)

Solutions A and B were mixed to give a final pH of 5.0, 5.5, or 6.0. Formulations were prepared by combining 0.272 parts stock LIF solution and 0.728 parts buffer to give a final LIF concentration of 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v.

Table 7 displays pH and osmolality (obtained using a Fiske One-Ten Osmometer) values for 0.4 and 1.0 mg/ml LIF samples prepared using the above buffer systems.

V. Freeze/Thaw Cycling

A. Sample Preparation and Methods

LIF samples were prepared by dilution of stock LIF (3.67 mg/ml in 2 mM phosphate buffer, pH 6.8) with acetate or citrate buffer containing sorbitol and polysorbate 80 to give a final buffer concentration of 10 mM, a theoretical pH of 4.0, 4.5, 5.0, 5.5, or 6.0, a final sorbitol concentration of 5% w/v, a final polysorbate 80 concentration of 0.01% w/v and a final LIF concentration of 1 mg/ml (see Section IV). The final pH of each sample was essentially the same as predicted by theory. Solutions (3 ml) were filtered through 0.22 m sterile filters (Millex GV) with the first 0.5 ml aliquot from the filter being retained as a separate sample for the preliminary determination of filter adsorption. Subsequent 0.5 ml aliquots were filtered into sterile 2 ml glass vials and capped with sterile rubber/teflon lined caps and crimped. One vial for each formulation was analysed on the day of preparation and all other vials were stored at -80°C. On each of 5 days, all vials were thawed and one vial of each formulation was centrifuged and an aliquot taken for dilution (in this study, all samples were analysed at a LIF concentration of 0.1 mg/ml) and analysis by RP, IEC, and SEC.

A 0.1 mg/ml standard solution was prepared by diluting the LIF stock solution with 2 mM phosphate buffer, pH 6.42 containing 0.01% polysorbate 80. This standard solution was

stored at 4°C for a total of 6 days and analysed along with each sample set.

B. Results

Figure 4 represents the individual peak areas for samples at each pH with concentration being expressed as a percentage of the initial concentration measured by each of the three methods. While there was some variability in the individual results (most likely due to the dilution step prior to analysis), there were no trends which would indicate loss of LIF upon freeze/thaw cycling.

Figure 5 illustrates the average concentration (as a percentage of the initial concentration) over 5 freeze/thaw cycles for each of the different pH values.

VI. Long Term Stability at -80°C, -20°C, 8°C and 25°C

A. Preparation of Samples for Storage at -80°C and -20°C

Five LIF formulations were prepared by dilution of stock LIF (3.67 mg/ml in 2 mM phosphate buffer, pH 6.42) with acetate or citrate buffer containing sorbitol and polysorbate 80 to give a final LIF concentration of 0.4 mg/ml or 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final polysorbate 80 concentration of 0.01% w/v (see Section V). The theoretical pH values were pH 4.0 (acetate buffer), 4.5 (acetate buffer), and 5.0 (citrate buffer). The final pH of each sample was essentially the same as predicted by theory.

Under aseptic conditions in a laminar flow cabinet, the formulations were sterile filtered using 0.22 m Millex GV (Millipore) filters. The first 1.0 ml of each filtrate was set aside and the vial marked accordingly (previous studies identified that approximately 1 ml was required to saturate the filter binding sites using Millex GV filter units). The remaining volume was filtered into a sterile 50 ml polypropylene tube. Aliquots of each formulation (1.15 ml/vial) were transferred using a multiple dispensing Eppendorf pipette with sterile tips into heat sterilised 2 ml glass vials and capped with sterile teflon lined rubber caps which were then

crimped. Vials were labelled and duplicate vials of each formulation were retained for the initial analysis. The remaining vials were stored at either -80°C or -20°C.

B. Preparation of Samples for Storage at 8°C and 25°C

Five LIF formulations were prepared by a dilution of stock LIF (3.67 mg/ml in 2 mM phosphate buffer, pH 6.42) with acetate or citrate buffer containing sorbitol and polysorbate 80 to give a final LIF concentration of 0.4 mg/ml or 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final polysorbate 80 concentration of 0.01% w/v. The theoretical pH values were pH 4.0 (acetate buffer), 4.5 (acetate buffer), and 5.0 (citrate buffer). The final pH of each sample was essentially the same as predicted by theory.

Formulations were filtered and filled into vials as described for the -80°C and -20°C samples. Samples were stored in temperature controlled incubators at either 8°C or 25°C. Incubators were checked daily to ensure the correct temperature was maintained.

C. Sample Analysis

All LIF samples were analysed undiluted according to the methods described in Section III. LIF standards at concentrations of 0.2, 0.4, 0.7 and 1.0 mg/ml were prepared from stock LIF (3.67 mg/ml in 2 mM phosphate buffer) by diluting with 2 mM phosphate buffer, pH 6.42 containing 0.01% w/v polysorbate 80. These standards were prepared fresh at the beginning of each set of analyses and were analysed along with the samples at the start and end of each analytical run.

At each time point, 2 vials were withdrawn from the freezers or incubators and approximately 200 μl was removed from each using a sterile 1 ml syringe and a sterile needle. These aliquots were placed into polypropylene autosampler vials and sealed with caps containing self-sealing septa to allow repeat injections from the same vial without evaporation.

Autosampler vials were transferred to the autosampler where they were maintained at 4°C

throughout the three analytical runs. The same sample and standard autosampler vials were used for each of the three analyses with the RP (10 μl injection volume) being conducted first, followed by the IEC (100 μl injection volume) and then the SEC (10 μl injection volume). The complete RP run took approximately 32 hours, and the IEC and SEC runs took approximately 25 hours each. It was assumed that any further degradation over this storage time in the autosampler would be minimal (standard solutions at pH 6.42 stored under the same conditions showed no change over the complete analytical period). Samples were analysed in the following order:

Blank x 2		
Standards	0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml and 1.0 mg/ml	
Blank		
0.4 mg/ml	Acetate pH 4.0	x2
	Acetate pH 4.5	x2
	Citrate pH 5.0	x2
1.0 mg/ml	Acetate pH 4.5	x2
	Citrate pH 5.0	x2
Blank		
Standards	0.2 mg/ml, 0.4 mg/ml, 0.7 mg/ml and 1.0 mg/ml	

Selected samples were also analysed for particulates using a Malvern Instruments Zetasizer 3000 particle size instrument. Samples were withdrawn from the storage vials using a syringe and placed in the sample cuvette. Samples were counted for 120 sec using a 200 m pinhole (to obtain the maximum signal), 90° scattering angle, and scattering source at 633 nm using a 10 mW He-Ne ion laser.

D. Results

Data pertaining to solution pH, LIF concentration in mg/ml (determined by comparison to LIF standard solutions), and the area % of the main peak relative to the total peak area for all LIF related peaks in the chromatogram analysed using the three chromatographic methods are shown in Tables 8 through 17. None of the samples showed significant shifts in pH over

the storage period.

1. Ion Exchange

Figures 6 through 15 illustrate IEC chromatograms for samples stored in each of the different buffer systems at 8 and 25°C. Two main products were evident for samples prepared in pH 4.0 and 4.5 buffers (eluting at approximately 9 and 10 min) whereas a single main product (eluting at approximately 10 min) was seen in the pH 5.0 samples. At each pH, there was evidence of several minor degradation products in the ion exchange chromatograms, however, due to inadequate resolution between the different products, the exact number of products could not be determined. Representative chromatograms for samples stored at -80 and -20°C are not shown as they were similar to the chromatograms at the higher temperatures with degradation products being present at significantly reduced levels.

The IEC results for samples stored at -80, -20, 8 and 25°C are shown graphically in Figures 16 through 18 with the main LIF peak plotted as a percentage of the total area for all LIF related peaks in the chromatogram as a function of storage time. The data illustrate the dependence of LIF stability on pH and temperature. The relative stability under each storage condition was similar for the 0.4 and 1.0 mg/ml formulations. The pH 4.0 samples displayed significant variability between the different time points at 8 and 25°C. Re-analysis of selected samples gave similar results to the original values. There was also evidence of degradation at pH 4.0 and 4.5 following storage at -20°C and -80°C. The stability was greatly improved at pH 5 in comparison to pH 4 and 4.5. After 55 days storage at 8°C, approximately 97% of the total peak area was present as the main LIF peak. Following storage at 25°C for 55 days, this value was reduced to approximately 78%. Samples prepared at pH 5 and stored at -80 or -20°C for up to 84 days showed no significant evidence of degradation.

2. Reversed Phase

Representative RP chromatograms are not included as all displayed essentially the same

elution characteristics (see Figure 1). In all cases, the chromatograms showed the presence of only one main peak eluting at approximately 36 min.

The RP results for samples stored at -80, -20, 8 and 25°C are shown graphically in Figures 19 through 21 with the measured concentration plotted as a function of storage time. The data illustrate the absence of significant change in the measured concentration over the storage period for each of the buffer and storage conditions utilised.

3. Size Exclusion

Representative SEC chromatograms are not included as all displayed essentially the same elution characteristics (see Figure 3). In all cases, the chromatograms showed the presence of one main peak eluting at approximately 26 min and a minor peak eluting at approximately 21 min.

The SEC results for samples stored at -80, -20, 8 and 25°C are shown graphically in Figures 22 through 24 with the measured concentration plotted as a function of storage time. The data illustrate the absence of significant change in the measured concentration over the storage period for each of the buffer and storage conditions utilised. Using the SEC method, there was no evidence of chain cleavage or crosslinking under the storage conditions studied.

4. Particle Size Analysis

Samples stored for 56 days at -80 and -20°C and for 41 days at 8 and 25°C were analysed for particulates using a laser light scattering instrument. All of the samples analysed displayed a count rate of "0 kCps" which effectively means that the samples contained no particulates (i.e. no signal was measurable).

VII. Summary

These studies demonstrated no notable loss of LIF following freeze thaw cycling of 1.0

mg/ml LIF solution formulations prepared in acetate or citrate buffers (pH 4 to 6) containing 5% w/v sorbitol and 0.01% w/v polysorbate 80. There was no significant loss of LIF on 0.2 m Sartorius Minisart filters when formulations were prepared at either 0.4 or 1.0 mg/ml in pH 5.0 or 5.5 citrate buffers containing 5% w/v sorbitol and 0.01% w/v polysorbate 80. For the pH 5.0 and 5.5 formulations, there was also no evidence of loss of LIF on the proposed vials, stoppers, or syringes.

At -80°C, there was no significant change in LIF concentrations measured by RP, IEC and SEC methods following storage for 84 days in the pH range of 4 to 5. At -20°C over the same time period, there was evidence of degradation for formulations prepared at pH 4 and analysed by IEC, but the remaining formulations were stable under these storage conditions. Generally, 0.4 and 1.0 mg/ml LIF formulations displayed similar stability characteristics under each of the conditions investigated. Formulations prepared at pH 5 were found to be stable for up to 8 weeks when stored at 8°C with minimal loss of the parent compound (~1%) shown by IEC and no loss shown by RP or SEC.

Table 1. Precision and Accuracy for the RP Assay

Nominal Conc. (mg/ml)	Total Peak Area	Measured Conc. (mg/ml)	Precision (CV, %)	Accuracy (% deviation)
0.4	14.213	0.391	0.44 (n=5)	-2.16
0.4	14.356	0.395		-1.21
0.4	14.361	0.395		-1.17
0.4	14.322	0.394		-1.43
0.4	14.255	0.392		-1.88
1.0	38.002	1.029	0.39 (n=5)	2.92
1.0	38.170	1.034		3.37
1.0	38.327	1.038		3.79
1.0	38.344	1.038		3.84
1.0	38.077	1.031		3.12

Table 2. Summary of RP Calibration Curves Over the Course of the Study

Date of Analysis	Slope	Intercept
21/3/97	33.460	-1.755
26/3/97	32.900	-0.312
2/4/97	34.491	-1.040
14/4/97	32.648	-0.137
18/4/97	32.865	1.006
22/4/97	32.865	0.566
29/4/97	33.705	1.092
6/5/97	34.617	0.535
16/5/97	35.920	0.113
20/5/97	35.666	-0.014
13/6/97	37.294	-0.382
mean	34.221	-0.030
SD	1.529	
CV,%	4.469	

Table 3. Precision and Accuracy for the IEC Assay

Nominal Conc. (mg/ml)	Total Peak Area	Measured Conc. (mg/ml)	Precision (CV, %)	Accuracy (% deviation)
0.4	8.310	0.397	0.68 (n=5)	-0.86
0.4	8.260	0.398		-0.62
0.4	8.265	0.399		-0.30
0.4	8.232	0.396		-1.10
0.4	8.234	0.403		0.65
1.0	21.929	1.007	0.41 (n=5)	0.70
1.0	21.910	1.005		0.51
1.0	21.918	1.008		0.77
1.0	21.901	1.004		0.35
1.0	21.870	1.014		1.43

Table 4. Summary of IEC Calibration Curves Over the Course of the Study

Date of Analysis	Slope	Intercept
22/3/97	2.953	-0.002
27/3/97	3.111	-0.038
4/4/97	3.104	-0.048
10/4/97	2.983	-0.019
16/4/97	2.987	-0.020
20/4/97	3.005	-0.018
24/4/97	2.942	-0.012
8/5/97	3.064	-0.055
18/5/97	3.005	-0.018
22/5/97	3.034	-0.036
15/6/97	3.137	-0.099
mean	3.030	-0.033
SD	0.066	---
CV,%	2.180	---

Table 5. Precision and Accuracy for the SEC Assay

Nominal Conc. (mg/ml)	Total Peak Area	Measured Conc. (mg/ml)	Precision (CV, %)	Accuracy (% deviation)
0.4	8.310	0.396	0.39 (n=5)	-0.98
0.4	8.260	0.394		-1.48
0.4	8.265	0.394		-1.46
0.4	8.232	0.393		-1.84
0.4	8.234	0.393		-1.86
1.0	21.929	1.002	0.11 (n=5)	0.23
1.0	21.910	1.001		0.11
1.0	21.918	1.001		0.15
1.0	21.901	1.000		0.07
1.0	21.870	0.999		-0.05

Table 6. Summary of SEC Calibration Curves Over the Course of the Study

Date of Analysis	Slope	Intercept
23/3/97	21.332	0.202
28/3/97	21.278	0.166
5/4/97	22.351	0.230
11/4/97	21.672	0.054
17/4/97	20.810	0.419
21/4/97	21.561	0.130
25/4/67	21.845	0.074
9/5/97	21.883	-0.090
19/5/97	21.963	0.158
23/5/97	21.794	-0.003
16/6/97	22.558	-0.474
mean	21.732	0.079
SD	0.491	----
CV,%	2.258	----

Table 7. pH and Osmolality of AM424 Formulations

buffer / theoretical pH	AM424 conc. (mg/ml)	measured pH	osmolality (mOsm/kg)
Acetate / pH 4.0	0.4	3.95	297
Acetate / pH 4.5	0.4	4.48	297
Citrate / pH 5.0	0.4	4.94	303
Acetate / pH 4.5	1.0	4.47	294
Citrate / pH 5.0	1.0	4.96	305

Table 8 Summary of 0.4 mg/ml, pH 4.0 AM424 Formulation Stability Following Storage at 8°C and 25°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.03	acetate	0.4	8	0	0.40, 0.39	100, 100	0.37, 0.37	98.9, 99.0	0.39, 0.40	98.8, 98.7
----				7	0.40, 0.40	100, 100	0.35, 0.34	91.7, 87.2	0.39, 0.40	97.6, 97.5
4.07				13	0.39, 0.39	100, 100	0.33, 0.37	90.8, 92.8	0.40, 0.40	98.9, 98.9
----				19	0.40, 0.40	100, 100	0.34, 0.33	89.7, 86.8	0.38, 0.38	98.8, 99.0
4.06				27	0.40, 0.40	100, 100	0.33, 0.33	84.6, 83.7	0.40, 0.40	98.9, 98.9
4.06				41	0.40, 0.40	100, 100	0.34, 0.35	86.9, 88.2	0.40, 0.41	98.9, 98.9
4.16				55	0.40, 0.41	100, 100	0.34, 0.33	89.2, 83.0	0.40, 0.40	99.0, 99.0
4.03	acetate	0.4	25	0	0.40, 0.39	100, 100	0.37, 0.37	98.9, 99.0	0.39, 0.40	98.8, 98.7
----				7	0.39, 0.39	100, 100	0.33, 0.36	85.1, 91.5	0.39, 0.40	97.3, 97.4
4.06				13	0.40, 0.39	100, 100	0.28, 0.30	74.7, 80.7	0.39, 0.41	99.2, 99.1
----				19	0.40, 0.39	100, 100	0.31, 0.32	78.3, 80.3	0.38, 0.38	99.0, 99.2
4.07				27	0.40, 0.40	100, 100	0.29, 0.30	73.3, 74.5	0.40, 0.40	99.4, 99.2
4.09				41	0.40, 0.40	100, 100	0.31, 0.31	76.1, 77.8	0.41, 0.41	99.2, 99.2
4.12				55	0.41, 0.40	100, 100	0.25, 0.24	62.6, 59.8	0.40, 0.40	99.3, 99.7

Underlined values represent repeat analyses

Table 9. Summary of 0.4 mg/ml, pH 4.0 AM424 Formulation Stability Following Storage at -80°C and -20°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
3.95	acetate	0.4	-80	0	0.41, 0.40	100, 100	0.38, 0.38	98.4, 98.6	0.40, 0.40	98.9, 98.5
3.98				28	0.41, 0.41	100, 100	0.38, 0.39	97.8, 98.8	0.39, 0.40	98.2, 98.0
3.99				56	0.41, 0.41	100, 100	0.37, 0.38	96.6, 98.9	0.39, 0.39	98.3, 98.3
4.05				84	0.43, 0.42	100, 100	0.40, 0.38	98.6, 99.1	0.41, 0.41	99.3, 98.6
3.95	acetate	0.4	-20	0	0.41, 0.40	100, 100	0.38, 0.38	98.4, 98.6	0.40, 0.40	98.9, 98.5
3.95				28	0.41, 0.42	100, 100	0.38, 0.39	96.9, 97.8	0.40, 0.40	98.7, 98.5
4.04				56	0.40, 0.41	100, 100	0.36, 0.36	94.2, 93.7	0.40, 0.40	99.0, 98.9
4.03				84	0.42, 0.43	100, 100	0.38, 0.38	92.5, 93.1	0.42, 0.41	99.2, 98.9

Table 10. Summary of 0.4 mg/ml, pH 4.5 AM424 Formulation Stability Following Storage at 8°C and 25°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.52	acetate	0.4	8	0	0.39, 0.39	100, 100	0.36, 0.36	99.0, 98.9	0.39, 0.38	98.8, 98.8
----				7	0.38, 0.38	100, 100	0.37, 0.36	95.4, 95.6	0.38, 0.39	97.7, 97.8
4.53				13	0.38, 0.38	100, 100	0.38, 0.36	98.3, 95.6	0.39, 0.38	99.0, 99.0
----				19	0.38, 0.38	100, 100	0.37, 0.35	97.8, 93.3	0.38, 0.38	98.2, 98.2
4.53				27	0.38, 0.38	100, 100	0.35, 0.36	90.8, 94.1	0.39, 0.39	98.9, 98.8
4.51				41	0.39, 0.39	100, 100	0.37, 0.36	95.3, 94.2	0.39, 0.39	98.9, 98.8
4.59				55	0.40, ----	100, ----	0.35, 0.33	89.6, 85.9	0.39, 0.39	99.0, 98.9
4.52	acetate	0.4	25	0	0.39, 0.39	100, 100	0.36, 0.36	99.0, 98.9	0.39, 0.39	98.8, 98.8
----				7	0.38, 0.38	100, 100	0.36, 0.34	94.7, 88.8	0.39, 0.39	98.1, 98.2
4.52				13	0.39, 0.38	100, 100	0.33, 0.35	86.8, 91.0	0.39, 0.38	99.0, 99.0
----				19	0.38, 0.38	100, 100	0.31, 0.30	82.0, 80.0	0.38, 0.38	99.1, 99.0
4.52				27	0.38, 0.38	100, 100	0.30, 0.29	75.8, 73.5	0.39, 0.39	99.1, 99.2
4.53				41	0.40, 0.40	100, 100	0.28, 0.28	71.2, 71.1	0.39, 0.39	99.2, 99.3
4.55				55	0.39, 0.40	100, 100	0.22, 0.24	53.4, 59.1	0.39, 0.39	99.3, 99.4

Underlined values represent repeat analyses

Table 11. Summary of 0.4 mg/ml, pH 4.5 AM424 Formulation Stability Following Storage at -80°C and -20°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.48	acetate	0.4	-80	0	0.40, 0.40	100, 100	0.39, 0.40	99.0, 98.9	0.40, 0.40	98.9, 98.8
4.49				28	0.41, 0.40	100, 100	0.39, 0.38	98.7, 98.7	0.40, 0.39	98.1, 98.0
4.49				56	0.40, 0.40	100, 100	0.38, 0.38	98.5, 98.6	0.39, 0.39	98.4, 98.2
4.55				84	0.42, 0.42	100, 100	0.40, 0.40	98.6, 98.4	0.42, 0.42	98.5, 98.5
4.48	acetate	0.4	-20	0	0.40, 0.40	100, 100	0.39, 0.40	99.0, 98.9	0.40, 0.40	98.9, 98.8
4.47				28	0.41, 0.41	100, 100	0.39, 0.38	98.8, 96.9	0.40, 0.40	98.4, 98.6
4.52				56	0.40, 0.40	100, 100	0.39, 0.38	98.5, 97.3	0.40, 0.39	98.6, 98.7
4.53				84	0.42, 0.42	100, 100	0.40, 0.40	96.4, 96.5	0.42, 0.42	99.0, 99.0

Table 12. Summary of 1.0 mg/ml, pH 4.5 AM424 Formulation Stability Following Storage at 8°C and 25°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.54	acetate	1.0	8	0	0.99, 0.99	100, 100	0.96, 0.96	98.5, 98.6	0.99, 0.99	98.6, 98.2
----				7	0.98, 0.98	100, 100	0.98, 0.99	96.9, 979	0.99, 0.99	98.6, 98.6
4.57				13	0.98, 0.99	100, 100	0.94, 0.96	96.2, 97.6	0.98, 0.98	98.8, 98.7
----				19	0.98, 1.00	100, 100	0.96, 0.94	97.5, 95.6	1.00, 0.99	98.6, 98.8
4.56				27	0.99, 1.00	100, 100	0.94, 0.89	97.0, 90.1	1.00, 1.00	98.6, 98.8
4.55				41	0.98, 0.99	100, 100	0.88, 0.90	90.3, 92.1	0.98, 0.98	98.9, 98.9
4.61				55	0.99, 1.00	100, 100	0.90, 0.85	91.2, 86.1	0.99, 0.99	98.9, 98.9
4.54	acetate	1.0	25	0	0.99, 0.99	100, 100	0.96, 0.96	98.5, 98.6	0.99, 0.99	98.6, 98.2
----				7	0.99,0.99	100, 100	0.92, 0.94	91.4, 92.7	0.99, 0.99	98.9, 98.9
4.57				13	1.00, 0.99	100, 100	0.82, 0.86	83.6, 88.6	0.98, 0.98	99.0, 99.0
----				19	1.00, 1.00	100, 100	0.84, 0.81	83.7, 80.9	1.00, 1.00	98.9, 98.9
4.57				27	1.00, 1.00	100, 100	0.78, 0.81	77.1, 79.3	1.00, 1.00	99.0, 99.0
4.59				41	0.99, 0.99	100, 100	0.68, 0.65	66.4, 63.9	0.98, 0.98	98.9, 99.1
4.61				55	1.00, 0.99	100, 100	0.59, 0.60	56.7, 58.9	0.99, 0.99	99.1, 99.1

Underlined values represent repeat analyses

Table 13. Summary of 1.0 mg/ml, pH 4.5 AM424 Formulation Stability Following Storage at -80°C and -20°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.47	acetate	1.0	-80	0	1.00, 1.00	100, 100	0.97, 0.98	98.9, 98.7	0.99, 0.99	98.7, 98.6
4.47				28	1.00, 1.00	100, 100	0.97, 0.97	98.4, 98.3	0.99, 0.99	98.4, 98.4
4.50				56	0.99, 0.98	100, 100	0.96, 0.95	98.5, 98.3	0.97, 0.97	98.5, 98.6
4.53				84	1.00, 1.00	100, 100	0.96, 0.96	98.3, 98.5	0.98, 0.98	98.6, 98.4
4.47	acetate	1.0	-20	0	1.00, 1.00	100, 100	0.97,0 98	98.9, 98.7	0.99, 0.99	98.7, 98.6
4.48				28	1.00, 0.99	100, 100	0.98, 0.97	98.3, 97.4	1.00, 0.99	98.5, 98.6
4.50				56	0.98, 0.99	100, 100	0.94, 0.96	97.4, 98.0	0.98, 0.98	98.6, 98.5
4.51				84	0.99, 0.99	100, 100	0.95, 0.97	96.9, 98.4	0.99, 0.99	98.7, 98.5

Table 14. Summary of 0.4 mg/ml, pH 5.0 AM424 Formulation Stability Following Storage at 8°C and 25°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
5.02	citrate	0.4	8	0	0.38, 0.37	100, 100	0.36, 0.36	98.8, 98.7	0.38, 0.38	98.6, 98.4
----				7	0.37, 0.37	100, 100	0.38, 0.38	98.4, 98.4	0.37, 0.37	98.1, 98.1
5.03				13	0.37, 0.37	100, 100	0.38, 0.38	98.4, 98.4	0.38, 0.38	98.6, 98.7
----				19	0.37, 0.37	100, 100	0.37, 0.37	98.4, 98.5	0.37, 0.37	98.3, 98.0
5.06				27	0.38, 0.38	100, 100	0.38, 0.37	98.4, 98.5	0.38, 0.38	98.6, 98.6
5.04				41	0.39, 0.39	100, 100	0.38, 0.37	97.8, 97.9	0.38, 0.38	98.7, 98.7
5.07				55	0.39, 0.39	100, 100	0.37, 0.37	97.7, 97.5	0.39, 0.38	98.8, 98.7
5.02	citrate	0.4	25	0	0.38, 0.37	100, 100	0.36, 0.36	98.8, 98.7	0.38, 0.38	98.6, 98.4
----				7	0.37, 0.37	100, 100	0.37, 0.36	97.0, 97.0	0.38, 0.38	98.7, 98.5
5.05				13	0.38, 0.37	100, 100	0.36, 0.37	95.4, 95.1	0.37, 0.37	98.7, 98.7
----				19	0.37, 0.37	100, 100	0.35, 0.35	93.8, 93.9	0.38, 0.38	98.8, 98.8
5.05				27	0.38, 0.38	100, 100	0.34, 0.34	92.0, 91.8	0.39, 0.39	99.3, 99.0
5.06				41	0.39, 0.39	100, 100	0.33, 0.34	87.0, 87.4	0.38, 0.38	99.1, 99.1
5.03				55	0.39, 0.39	100, 100	0.30, 0.30	77.8, 77.9	0.39, 0.39	99.0, 98.9

Underlined values represent repeat analyses

Table 15. Summary of 0.4 mg/ml, pH 5.0 AM424 Formulation Stability Following Storage at -80°C and -20°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.94	citrate	0.4	-80	0	0.41, 0.40	100, 100	0.40, 0.40	98.9, 98.8	0.39, 0.39	98.8, 98.7
4.98				28	0.41, 0.41	100, 100	0.39, 0.39	98.5, 98.5	0.39, 0.39	98.3, 98.3
4.98				56	0.40, 0.40	100, 100	0.38, 0.38	98.6, 98.6	0.38, 0.38	98.4, 98.3
5.00				84	0.42, 0.42	100, 100	0.40, 0.40	98.7, 98.4	0.41, 0.41	98.5, 98.5
4.94	citrate	0.4	-20	0	0.41, 0.41	100, 100	0.40, 0.40	98.9, 98.8	0.39, 0.39	98.8, 98.7
4.95				28	0.41, 0.41	100, 100	0.39, 0.39	98.5, 98.5	0.40, 0.40	98.5, 98.6
4 96				56	0.40, 0.40	100, 100	0.38, 0.39	98.4, 98.6	0.39, 0.39	98.6, 98.6
4.97				84	0.42, 0.42	100, 100	0.41, 0.41	98.6, 98.7	0.41, 0.41	99.0, 98.8

Table 16. Summary of 1.0 mg/ml, pH 5.0 AM424 Formulation Stability Following Storage at 8°C and 25°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
5.00	citrate	1.0	8	0	0.98, 0.98	100, 100	0.95, 0.95	98.5, 98.5	0.97, 0.97	98.2, 98.1
----				7	0.98, 0.98	100, 100	0.99, 0.99	98.5, 98.5	0.97, 0.98	98.5, 98.5
5.05				13	0.97, 0.97	100, 100	0.94, 0.94	98.1, 98.2	0.96, 0.96	98.2, 98.0
----				19	0.99, 0.99	100, 100	0.95, 0.95	98.1, 98.0	0.98, 0.98	98.5, 98.6
5.02				27	0.98, 0.99	100, 100	0.99, 0.98	98.0, 98.1	0.98, 0.98	98.6, 98.6
5.04				41	0.96, 0.96	100, 100	0.94, 0.94	97.5, 97.6	0.95, 0.96	98.7, 98.6
5.04				55	0.98, 0.98	100, 100	0.94, 0.94	97.0, 97.2	0.97, 0.98	98.6, 98.8
5.00	citrate	1.0	25	0	0.98, 0.98	100, 100	0.95, 0.95	98.5, 98.5	0.97, 0.97	98.2, 98.1
----				7	0.97, 0.97	100, 100	0.97, 0.97	97.0, 97.0	0.98, 0.98	98.8, 98.6
5.06				13	0.98. 0.97	100, 100	0.92, 0.91	94.6, 94.7	0.97, 0.97	98.8, 98.8
----				19	0.99, 1.00	100, 100	0.90, 0.89	92.2, 92.3	0.98, 0.98	98.8, 98.6
5.05				27	0.99, 0.99	100, 100	0.91, 0.91	90.3, 90.3	0.99, 0.98	98.8, 98.8
5.06				41	0.97, 0.97	100, 100	0 80, 0.80	83.0, 83.0	0.96, 0.96	98.6, 98.7
5.00				55	0.99, 0.98	100, 100	0.76, 0.76	77.7, 78.0	0.99, 0.97	99.0, 98.7

Underlined values represent repeat analyses

Table 17. Summary of 1.0 mg/ml, pH 5.0 AM424 Formulation Stability Following Storage at -80°C and -20°C

measured pH	buffer	Nominal AM424 Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
4.96	citrate	1.0	-80	0	1.00, 1.00	100, 100	0.98, 0.99	98.8, 98.8	0.98, 0.98	98.1, 98.1
4.97				28	1.00, 0.99	100, 100	0.96, 0.96	98.2, 98.1	0.98, 0.98	98.4, 98.4
4.95				56	0.97, 0.97	100, 100	0.95, 0.95	98.4, 98.4	0.96, 0.96	98.5, 98.4
4.97				84	0.99, 0.99	100, 100	0.96, 0.96	98.4, 98.5	0.97, 0.97	98.5, 98.5
4.96	citrate	1.0	-20	0	1.00, 1.00	100, 100	0.98, 0.98	98.8, 98.8	0.98, 0.98	98.1, 98.1
4.96				28	0.99, 1.00	100, 100	0.97, 0.97	98.3, 98.2	0.98, 0.98	98.5, 98.4
4.94				56	0.98, 0.97	100, 100	0.95, 0.95	98.3, 98.3	0.97, 0.96	98.6, 98.5
4.96				84	0.99, 0.99	100, 100	0.96, 0.96	98.4, 98.3	0.97, 0.96	98.5, 98.6

Example 2.

I. Analytical Methods

A. Reversed Phase (RP), Ion Exchange (IE) and Size Exclusion (SEC) Assays were conducted as described in Example 1.

II. Buffer Composition

All LIF samples were prepared by dilution of stock LIF solution containing 3.67 mg/ml LIF in 2 mM phosphate buffer, pH 6.42 to give the desired final LIF concentration (either 0.4 or 1.0 mg/ml) and composition of buffer components. The final composition of each solution contained 10 mM citrate buffer, 5% w/v sorbitol and 0.01% w/v Polysorbate 80. Samples differed in the final concentration of phosphate buffer (present from the original stock LIF solution) depending on the dilution factor. The 0.4 mg/ml LIF solutions contained 0.22 mM residual phosphate while the 1.0 mg/ml LIF solutions contained 0.54 mM residual phosphate. The composition of each buffer was as follows:

A. Citrate Buffer for 0.4 mg/ml LIF Formulations

Solution A: 11.22 mM sodium citrate dihydrate (Merck #1.06448)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 11.22 mM citric acid monohydrate (Merck #1.00244)
5.61% w/v sorbitol (Sigma Chemicals #S1876)
0.0112% Polysorbate 80 (Sigma Chemicals #P1754)

Solutions A and B were mixed to give a final pH of 5.5. Formulations were prepared by combining 0.109 parts stock LIF solution and 0.891 parts buffer to give a final LIF concentration of 0.4 mg/ml, a final buffer concentration of 10 mM, a final sorbitol

concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v. The measured osmolality of the final 0.4 mg/ml LIF formulation was 317 mOsm/kg.

B. Citrate Buffer for 1.0 mg/ml LIF Formulations

Solution A: 13.75 mM sodium citrate (Merck #1.06448)
6.88% w/v sorbitol (Sigma Chemicals #S1876)
0.0138% w/v Polysorbate 80 (Sigma Chemicals #P1754)

Solution B: 13.75 mM citric acid (Merck #1.00244)
6.88% w/v sorbitol (Sigma Chemicals S1876)
0.0138% w/v Polysorbate 80 (Sigma Chemicals P1754)

Solutions A and B were mixed to give a final pH of 5.5. Formulations were prepared by combining 0.272 parts stock LIF solution and 0.728 parts buffer to give a final LIF concentration of 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final Polysorbate 80 concentration of 0.01% w/v. The measured osmolality of the final 1.0 mg/ml LIF formulation was 322 mOsm/kg.

II. Long Term Stability at 8°C and 25°C

A. Preparation of Samples for Storage at 8°C and 25°C

LIF formulations were prepared by dilution of stock LIF (3.67 mg/ml in 2 mM phosphate buffer, pH 6.42) with citrate buffer containing sorbitol and polysorbate 80 to give a final LIF concentration of 0.4 mg/ml or 1.0 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v and a final polysorbate 80 concentration of 0.01% w/v (see Section II). The theoretical pH was 5.5 and the actual pH of each sample was measured and recorded.

Under aseptic conditions in a laminar flow cabinet, the formulations were sterile filtered

using 0.22 m Millex GV (Millipore) filters. The first 1.15 ml of each filtrate was set aside and the vial marked accordingly. The remaining volume was filtered into a sterile 50 ml polypropylene tube. Aliquots of each formulation (1.15 ml/vial) were transferred using a multiple dispensing Eppendorf pipette with sterile tips into heat sterilised 2 ml glass vials and capped with sterile teflon lined rubber caps which were then crimped. Vials were labelled and duplicate vials of each formulation were retained for the initial analysis. The remaining vials were stored at either 8°C or 25°C.

B. Sample Analysis

All LIF samples were analysed undiluted along with standards according to the methods described in Example 1. At each time point, 2 vials were withdrawn from the incubators and approximately 200 μl was removed from each using a sterile 1 ml syringe and a sterile needle. These aliquots were placed into polypropylene autosampler vials and sealed with caps containing self-sealing septa to allow repeat injections from the same vial without evaporation. The original glass sample vials were then marked with the time point and placed at -80°C for repeat analysis (if required) or use in other studies.

Autosampler vials were transferred to the autosampler where they were maintained at 4°C throughout the three analytical runs. The same sample and standard autosampler vials were used for each of the three analyses with the RP (10 μl injection volume) being conducted first, followed by the IEC (100 μl injection volume) and then the SEC (10 μl injection volume). The complete RP run took approximately 20 hours, and the IEC and SEC runs took approximately 15 hours each. It was assumed that any further degradation over this storage time in the autosampler would be minimal (standard solutions at pH 6.42 stored under the same conditions showed no change over the complete analytical period).

Selected samples were also analysed for particulates using a Malvern Instruments Zetasizer 3000 particle size instrument. Samples were withdrawn from the storage vials using a syringe

and placed in the sample cuvette. Samples were counted for 120 sec using a 200 m pinhole (to obtain the maximum signal), 90° scattering angle, and scattering source at 633 nm using a 10 mW He-Ne ion laser.

IV. Results

Data pertaining to solution pH, LIF concentration in mg/ml (determined by comparison to LIF standard solutions), and the area % for the main peak relative to the total peak area for all LIF related peaks in the chromatogram analysed using the three chromatographic methods are shown in Tables 18 and 19. For each set of samples, there was a slight decrease in solution pH of approximately 0.1 unit over the 92 day storage period.

1. Ion Exchange

Figures 25 through 28 illustrate ion exchange chromatograms for samples stored at 8 and 25°C. A single main product (eluting at approximately 9 min) was seen in all samples. There was evidence of several minor degradation products in the ion exchange chromatograms, however, due to inadequate resolution between the different products, the exact number of products could not be determined. Figures 29 and 30 show a comparison of chromatograms for samples prepared at pH 5.0 (initial study) and those at pH 5.5 (this study) stored at 8°C and 25°C for 8 weeks. Chromatograms were normalised with respect to the retention time for the main peak to take into account slight changes in the chromatography between the two studies. In each case, the product distribution was similar with a higher proportion of the main degradation product noted in the pH 5.5 samples relative to the pH 5.0 samples.

The IEC results are shown graphically in Figure 31 with the main LIF peak plotted as a percentage of the total area for all LIF related peaks in the chromatogram as a function of storage time. The data illustrate the dependence of LIF stability on temperature. The relative stability under each storage condition was similar for the 0.4 and 1.0 mg/ml formulations.

After 92 days storage at 8°C, 95-96% of the total peak area was present as the main LIF peak. Following storage at 25°C for 92 days, this value was reduced to approximately 56-58%.

Figures 32 and 33 compare the IEC stability data (main peak area expressed as a percentage of the total) obtained for samples at pH 5.5 with that from the previous study with samples prepared at pH 5.0. At 25°C, a slight increase in the rate of degradation was evident at pH 5.5.

Figures 34 through 37 illustrate the concentration of LIF as a function of time as determined by IEC as well as RP and SEC methods.

2. Reversed Phase

RP chromatograms are not included in this report as all displayed essentially the same elution characteristics. In all cases, the chromatograms showed the presence of only one main peak eluting at approximately 36 min.

The RP results are shown graphically in Figures 34 through 37 with the measured concentration plotted as a function of storage time. The data illustrate the absence of significant change in the measured concentration over the storage period.

3. Size Exclusion

SEC chromatograms are not included in this report as all displayed essentially the same elution characteristics. In all cases, the chromatograms showed the presence of one main peak eluting at approximately 25 min and a minor peak eluting at approximately 21 min.

The SEC results are shown graphically in Figures 34 through 37 with the measured concentration plotted as a function of storage time. The data illustrate the absence of significant change in the measured concentration over the storage period. Using the SEC method, there was no evidence of chain cleavage or crosslinking under the storage conditions studied.

4. Particle Size Analysis

Samples stored for 102 days at 8 and 25°C were analysed for particulates using a laser light scattering instrument. All of the samples analysed displayed a count rate of "0-0.5 kCps" which effectively means that the samples contained no particulates (i.e. no signal was measurable).

V. Summary

These studies demonstrated that formulations prepared at pH 5.5 were stable for up to 13 weeks when stored at 8°C with loss of the parent compound being approximately 3% as shown by IEC. After storage for 56 days at 8°C, the loss of LIF was approximately 2% in comparison to approximately 1% for pH 5.0 samples stored under the same conditions (data from the initial study). At 25°C, the rate of degradation at pH 5.5 was significantly increased with approximately 12% loss occurring in 4 weeks. In comparison, pH 5.0 samples showed a decrease in LIF concentration of approximately 7-9% after 4 weeks at 25°C. As in the initial study, no loss of LIF was detected by RP or SEC under any of the conditions studied.

Table 18 Summary of AM424 Stability for 0.4 mg/ml Formulations at pH 5.5 Following Storage at 8°C and 25°C

Measured pH	Buffer	Nominal LIF Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc. (mg/ml)	SEC - Main Peak (area %)
5.61	citrate	0.4	8	0	0.42, 0.44	100, 100	0.40, 0.40	98.6, 98.6	0.40, 0.40	98.7, 98.7
5.56				14	0.38, 0.38	100, 100	0.37, 0.37	97.9, 98.0	0.39, 0.39	98.7, 98.7
5.59				29	0.41, 0.41	100, 100	0.39, 0.39	98.4, 98.3	0.41, 0.41	98.8, 98.7
5.51				42	0.40, 0.41	100, 100	0.37, 0.38	98.1, 97.8	0.40, 0.39	98.6, 98.7
5.47				56	0.39, 0.39	100, 100	0.39, 0.39	97.3, 97.1	0.40, 0.40	98.6, 98.7
5.48				77	0.39, 0.40	100, 100	0.38, 0.38	96.2, 96.3	0.39, 0.39	98.9, 98.9
5.48				92	0.42, 0.40	100, 100	0.37, 0.37	95.7, 95.8	0.38, 0.38	98.6, 98.7
5.61	citrate	0.4	25	0	0.42, 0.44	100, 100	0.40, 0.40	98.6, 98.6	0.40, 0.40	98.7, 98.7
5.57				14	0.38, 0.39	100, 100	0.35, 0.35	92.8, 92.8	0.39, 0.39	98.8, 98.9
5.59				29	0.41, 0.42	100, 100	0.35, 0.35	86.9, 86.8	0.41, 0.41	98.9, 99.0
5.52				42	0.41, 0.41	100, 100	0.31, 0.32	81.0, 81.8	0.38, 0.40	98.8, 99.0
5.48				56	0.39, 0.39	100, 100	0.29, 0.29	71.6, 71.9	0.41, 0.40	99.2, 99.0
5.48				77	0.41, 0.40	100, 100	0.26, 0.26	63.8, 64.0	0.40, 0.39	99.3, 99.1
5.48				92	0.40, 0.42	100, 100	0.23, 0.23	57.4, 57.7	0.39, 0.40	98.9, 99.2

Table 19 Summary of AM424 Stability for 1.0 mg/ml Formulations at pH 5.5 Following Storage at 8°C and 25°C

Measured pH	Buffer	Nominal LIF Conc. (mg/ml)	Storage Temp. (C)	Storage Time (days)	RP - Measured Conc. (mg/ml)	RP - Main Peak (area %)	IEC - Measured Conc. (mg/ml)	IEC - Main Peak (area %)	SEC - Measured Conc (mg/ml)	SEC - Main Peak (area %)
5.61	citrate	1.0	8	0	1.09, 1.08	100, 100	1.00, 1.00	98.6, 98.6	1.01, 1.01	98.6, 98.6
5.58				14	0.98, 0.99	100, 100	0.96, 0.96	97.7, 97.7	0.99, 0.99	98.6, 98.7
5.61				29	1.01, 1.02	100, 100	0.99, 0.99	97.5, 97.6	1.01, 1.01	98.5, 98.5
5.57				42	1.00, 1.01	100, 100	0.97, 0.97	97.3, 97.2	0.99, 0.98	98.4, 98.6
5.54				56	1.00, 0.99	100, 100	0.96, 0.96	96.8, 96.6	1.02, 1.02	98.4, 98.5
5.52				77	1.03, 1.02	100, 100	0.94, 0.94	96.0, 95.9	0.98, 0.99	98.5, 98.5
5.52				92	1.06, 1.04	100, 100	0.95, 0.94	95.3, 95.3	0.98, 0.98	98.4, 98.4
5.61	citrate	1.0	25	0	1.09, 1.08	100, 100	1.00, 1.00	98.6, 98.6	1.01, 1.01	98.6, 98.6
5.58				14	0.98, 0.98	100, 100	0.90, 0.90	91.7, 91.8	0.99, 0.99	98.7, 98.7
5.62				29	1.02, 1.01	100, 100	0.87, 0.87	85.6, 85.7	1.02, 1.02	98.7, 98.7
5.59				42	1.02, 1.01	100, 100	0.80, 0.80	80.0, 79.8	0.98, 0.98	98.8, 98.8
5.54				56	0.99, 1.02	100, 100	0.71, 0.71	68.9, 69.2	1.02, 1.03	98.8, 98.7
5.53				77	1.02, 1.03	100, 100	0.64, 0.64	61.0, 61.6	0.98, 0.98	98.9, 98.8
5.52				92	1.04, 1.09	100, 100	0.59, 0.58	56.0, 56.1	0.99, 0.99	98.8, 98.7

Example 3.

I. Sample Preparation

8°C and 25°C LIF Samples

LIF formulations were prepared by a dilution of stock LIF (3.67 mg/ml in 2 mM phosphate buffer) with citrate buffer containing sorbitol or NaCl to give a final LIF concentration of 0.05 or 0.4 mg/ml, a final buffer concentration of 10 mM, a final sorbitol concentration of 5% w/v or a final NaCl concentration of 0.9% w/v. The theoretical pH was 5.0 in all cases. Formulations were prepared and filled into vials as described previously.

II. Analytical Methods

Samples and standards were prepared as previously described. Analyses were conducted by RP and SEC and IEC was conducted using the Polycat A column.

The RP and SEC assays were the same as those described in Example 1. The IEC assay was conducted using a PolyLC PolyCAT A cation exchange, pH 6 phosphate buffer and a salt gradient. Detection was at 215 nm.

III. Results

Ion Exchange

IEC data for 0.4 mg/ml formulations are shown in Tables 20 and 21 and Figures 38 and 39 with the main peak expressed as % of the initial since the % of total area values differ for the Pharmacia and Polycat A columns. Raw data is shown in the table. At 25°C, the most stable formulations were the pH 5.0 citrate buffer containing sorbitol and Tween 80 and the pH 5.0 citrate containing NaCl. The least stable was the pH 5 citrate buffer containing only sorbitol and pH 5.5 citrate containing sorbitol and Tween 80 was somewhere in the middle.

SEC

SEC data for 0.05 and 0.4 mg/ml formulations are shown in Figures 40 and 41 with the main peak expressed as a % of the total area. There was some variability in the 0.05 mg/ml samples most likely due to the low concentration. There were no real trends for either buffer at 8°C or 25°C.

Freeze-Thaw Cycling

Freeze-thaw cycling studies for pH 5 citrate buffers containing sorbitol or NaCl and analysed by SEC are shown in Figures 42 to 44. After the 5th cycle there was a trend toward a decrease in the main peak as a % of the total area and a slight increase in the pre-eluting high molecular weight peak.

Table 20 AM424 0.4 mg/ml Stability Following Storage at 8°C Measured by IEC

Storage Time (weeks)	Citrate/Sorbitol /Tween pH 5.0 Measured Conc. (mg/ml)[a]	Citrate/Sorbitol /Tween pH 5.0 Main Peak (area%)[a]	Citrate/Sorbitol /Tween pH 5.5 Measured Conc. (mg/ml)[a]	Citrate/Sorbitol /Tween pH 5.5 Main Peak (area%)[a]	Citrate/Sorbitol pH 5.0 Measured Conc. (mg/ml)[b]	Citrate/Sorbitol pH 5.0 Main Peak (area%)[b]	Citrate/NaCl pH 5.0 Measured Conc. (mg/ml)[b]	Citrate/NaCl pH 5.0 Main Peak (area %)[b]
0	0.36, 0.36	98.8, 98.7	0.40, 0.40	98.6, 98.6	0.28, 0.28	73.1, 73.2	0.28, 0.28	72.9, 72.8
2	0.38, 0.38	98.4, 98.4	0.37, 0.37	97.9, 98.0	0.27, 0.28	72.1, 72.0	0.27, 0.28	71.9, 72.4
4	0.38, 0.37	98.4, 98.5	0.39, 0.39	98.4, 98.3	0.29, 0.28	73.3, 72.8	0.28, 0.28	74.1, 73.9
6	0.38, 0.37	97.8, 97.9	0.37, 0.38	98.1, 97.8	0.30, 0.29	73.4, 72.4	0.30, 0.30	73.9, 73.4
8	0.37, 0.37	97.7, 97.5	0.39, 0.39	97.3, 97.1	0.29, 0.29	71.9, 71.7	0.28, 0.29	71.6, 72.3

[a] Pharmacia Mono S Column
[b] PolyCAT A Column

Table 21 AM424 0.4 mg/ml Stability Following Storage at 25°C Measured by IEC

Storage Time (weeks)	Citrate/Sorbitol /Tween pH 5.0 Measured Conc. (mg/ml)[a]	Citrate/Sorbitol /Tween pH 5.0 Main Peak (area%)[a]	Citrate/Sorbitol /Tween pH 5.5 Measured Conc. (mg/ml)[a]	Citrate/Sorbitol /Tween pH 5.5 Main Peak (area%)[a]	Citrate/Sorbitol pH 5.0 Measured Conc. (mg/ml)[b]	Citrate/Sorbitol pH 5.0 Main Peak (area%)[b]	Citrate/NaCl pH 5.0 Measured Conc. (mg/ml)[b]	Citrate/NaCl pH 5.0 Main Peak (area %)[b]
0	0.36, 0.36	98.8, 98.7	0.40, 0.40	98.6, 98.6	0.28, 0.28	73.1, 73.2	0.28, 0.28	72.9, 72.8
2	0.36, 0.37	95.4, 95.1	0.35, 0.35	92.8, 92.8	0.26, 0.26	64.5, 67.9	0.27, 0.25	69.7, 67.4
4	0.34, 0.34	92.0, 91.8	0.35, 0.35	86.9, 86.8	0.24, 0.25	61.3, 61.6	0.26, 0.25	67.3, 66.4
6	0.33, 0.34	87.0, 87.4	0.31, 0.32	81.0, 81.8	0.24, 0.24	58.9, 59.0	0.26, 0.26	62.7, 62.9
8	0.30, 0.30	77.8, 77.9	0.29, 0.29	71.6, 71.9	0.21, 0.20	54.0, 51.7	0.24, 0.24	59.5, 59.2

[a] Pharmacia Mono S Column
[b] PolyCAT A Column

Example 4.

Preferred compositions comprise:

- LIF in a concentration of 400 to 1000 μg/ml
- pH of about 4.0 - 6.0
- surfactant
- isotonicity agent
- buffer.

Particularly preferred compositions are those wherein the pH range is about 4.5 - 5.5.

Example 5.

A particularly preferred composition comprises:

- LIF in a concentration of 400 to 1000 μg/ml
- pH of about 5.0
- 5% sorbitol
- 0.01% polysorbate 80
- citrate or acetate buffer.

DATED this 26th day of November, 1997

AMRAD Operations Pty Ltd

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicant

Figure 1.
Representative Reversed Phase Chromatogram
for LIF 1.0 mg/ml Standard Solution

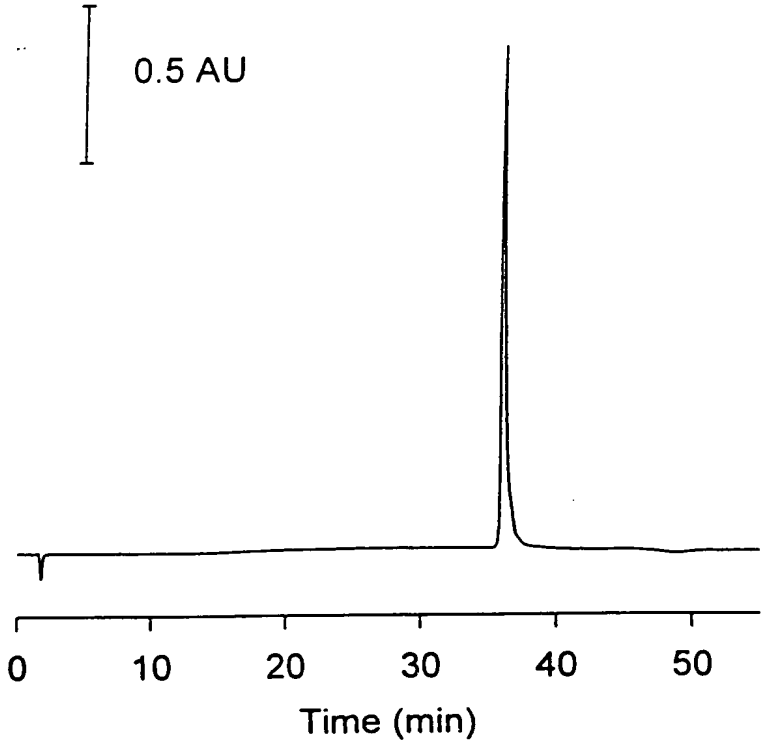


Figure 2

Representative Ion Exchange Chromatogram for LIF 1.0 mg/ml Standard Solution

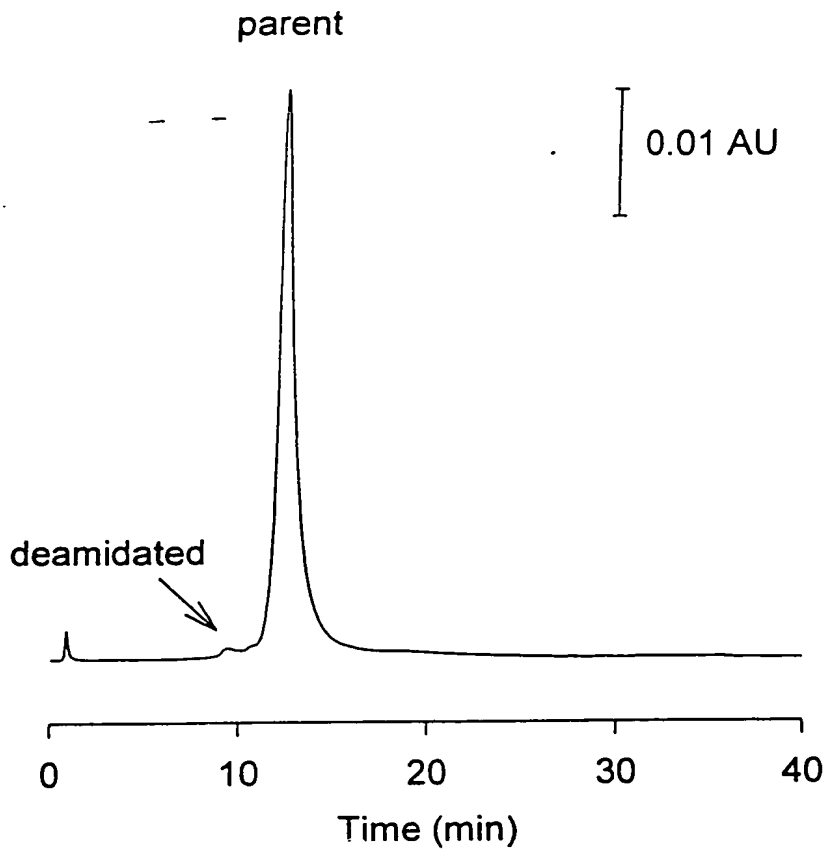


Figure 3

Representative Size Exclusion Chromatogram for LIF 1.0 mg/ml Standard Solution

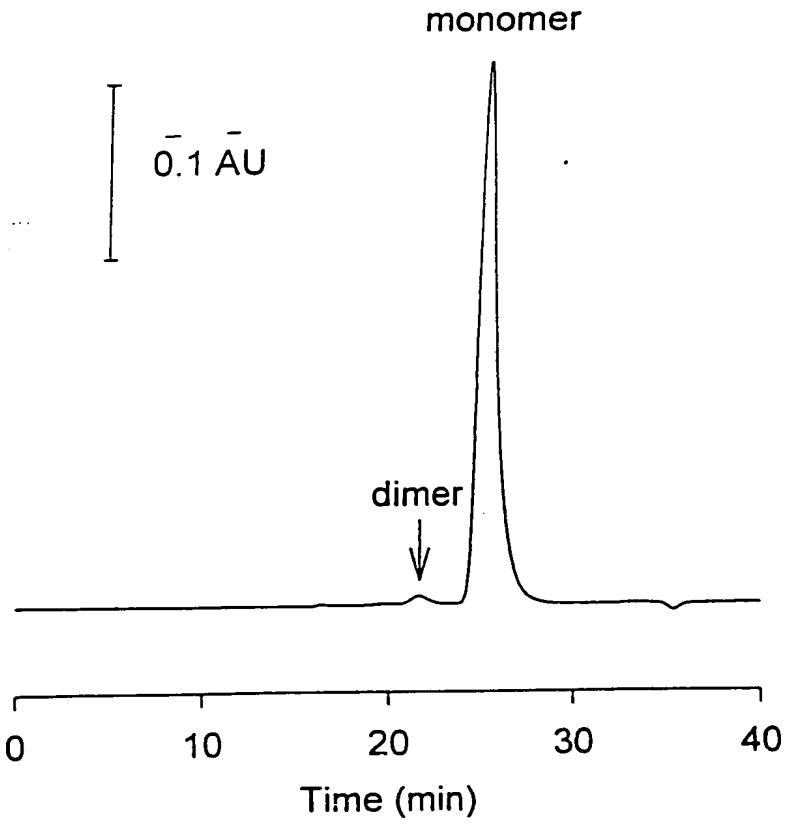


Figure 4
Individual Freeze/Thaw Cycling Results

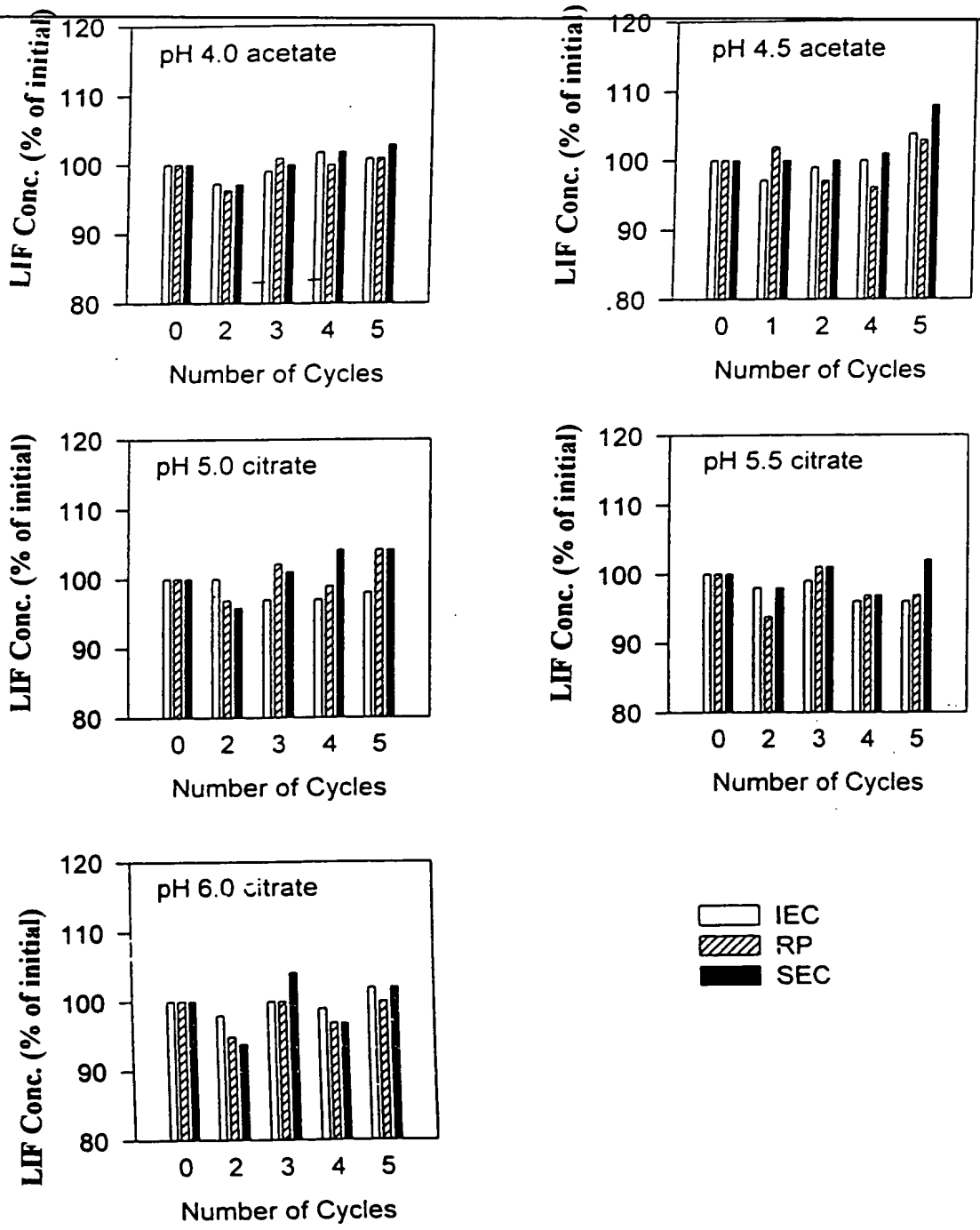


Figure 5

Average LIF Concentration Over 5 Freeze/Thaw Cycles (+/- SD)

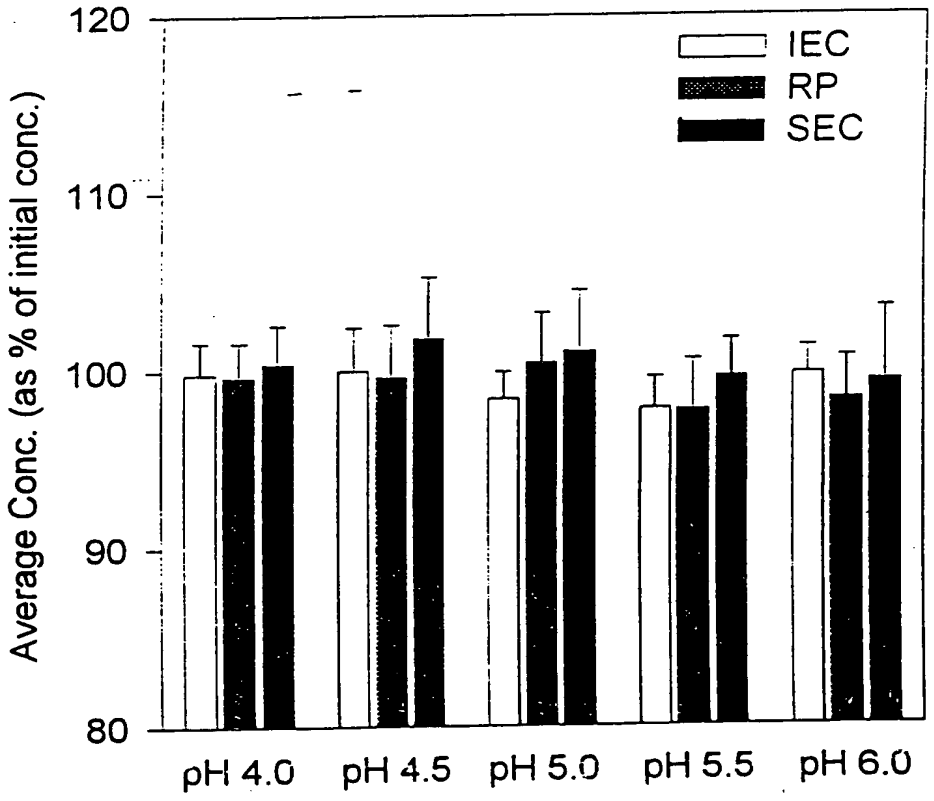


Figure 6

IEC Chromatograms for 0.4 mg/ml LIF in pH 4.0 Acetate Buffer Following Storage at 8°C

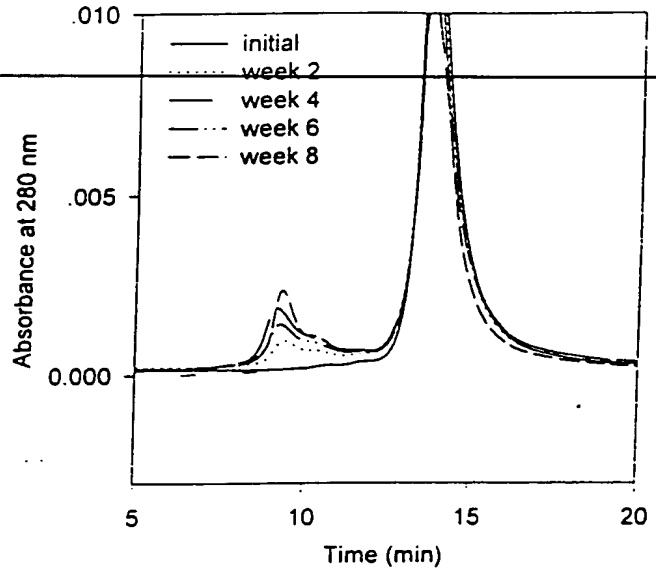


Figure 7

IEC Chromatograms for 0.4 mg/ml LIF in pH 4.0 Acetate Buffer Following Storage at 25°C

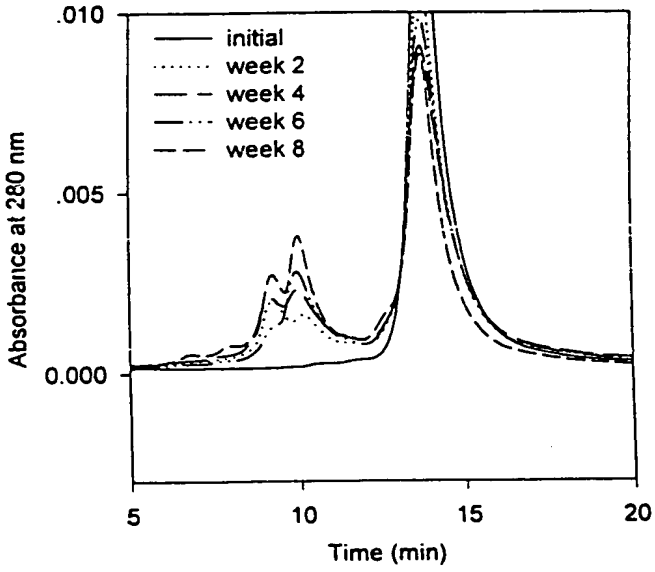


Figure 8

IEC Chromatograms for 0.4 mg/ml LIF in pH 4.5 Acetate Buffer Following Storage at 8°C

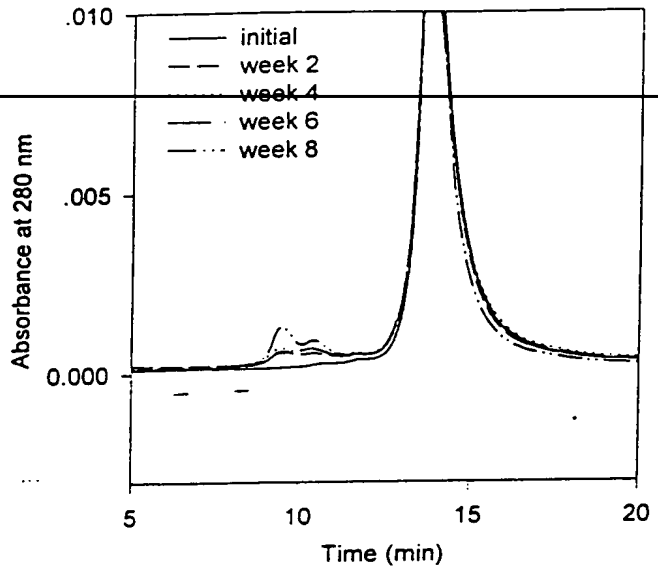


Figure 9

IEC Chromatograms for 0.4 mg/ml LIF in pH 4.5 Acetate Buffer Following Storage at 25°C

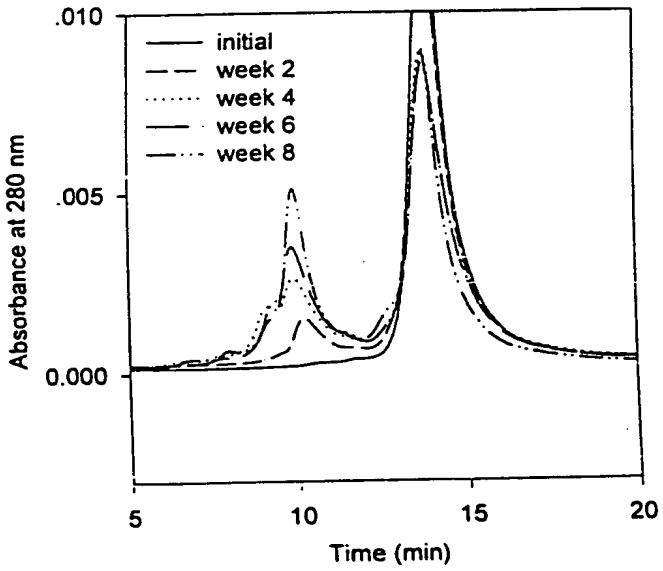


Figure 10

IEC Chromatograms for 1.0 mg/ml LIF in pH 4.5 Acetate Buffer Following Storage at 8°C

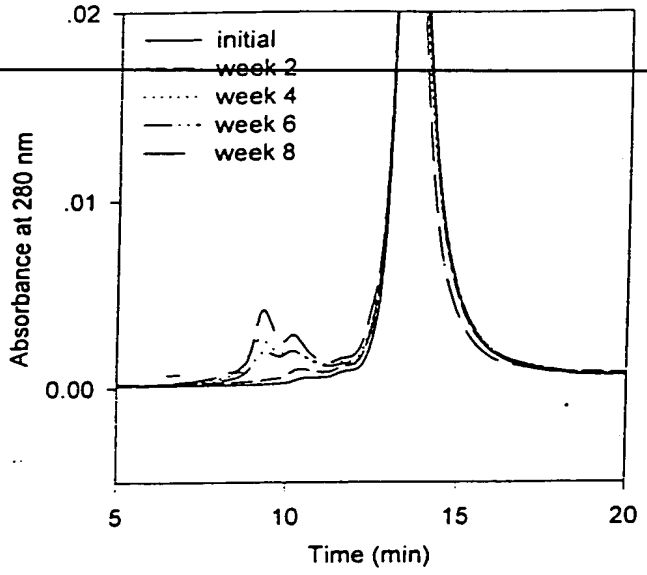


Figure 11

IEC Chromatograms for 1.0 mg/ml LIF in pH 4.5 Acetate Buffer Following Storage at 25°C

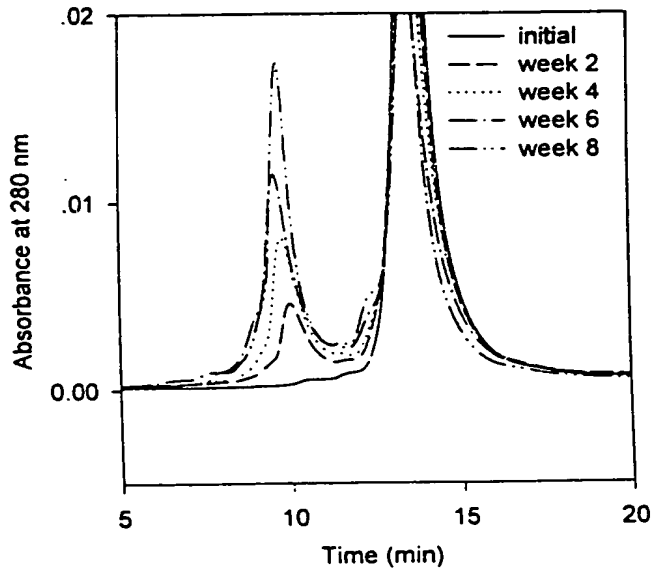


Figure 12

IEC Chromatograms for 0.4 mg/ml LIF in pH 5.0 Citrate Buffer Following Storage at 8°C

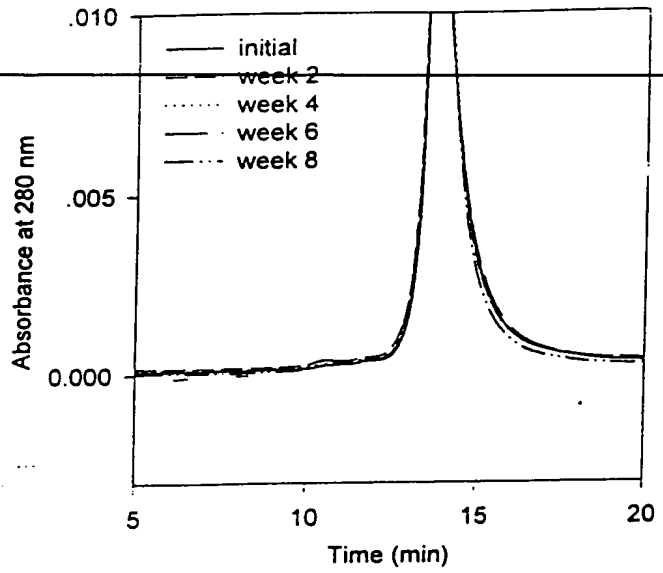


Figure 13

IEC Chromatograms for 0.4 mg/ml LIF in pH 5.0 Citrate Buffer Following Storage at 25°C

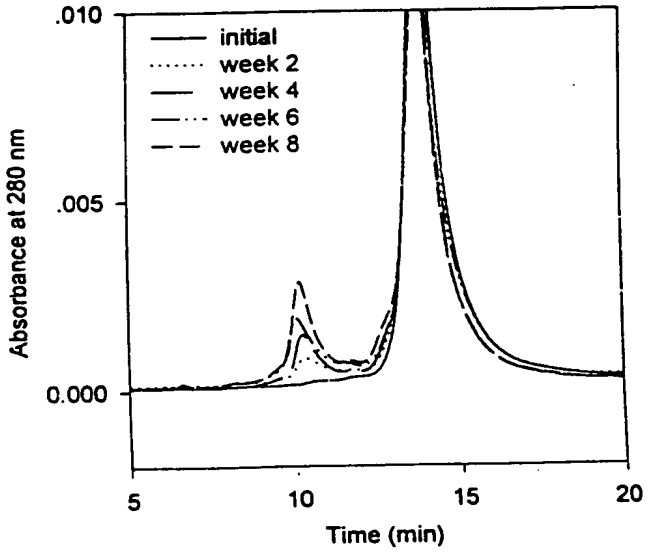


Figure 14

IEC Chromatograms for 1.0 mg/ml LIF in pH 5.0 Citrate Buffer Following Storage at 8°C

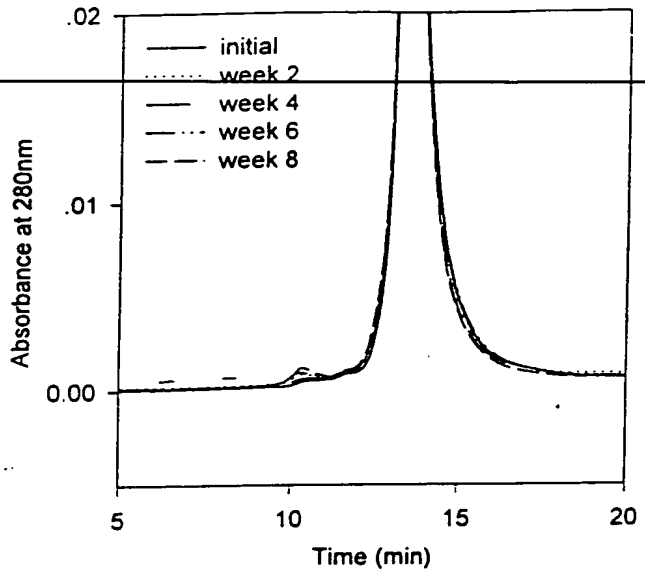


Figure 15

IEC Chromatograms for 1.0 mg/ml LIF in pH 5.0 Citrate Buffer Following Storage at 25°C

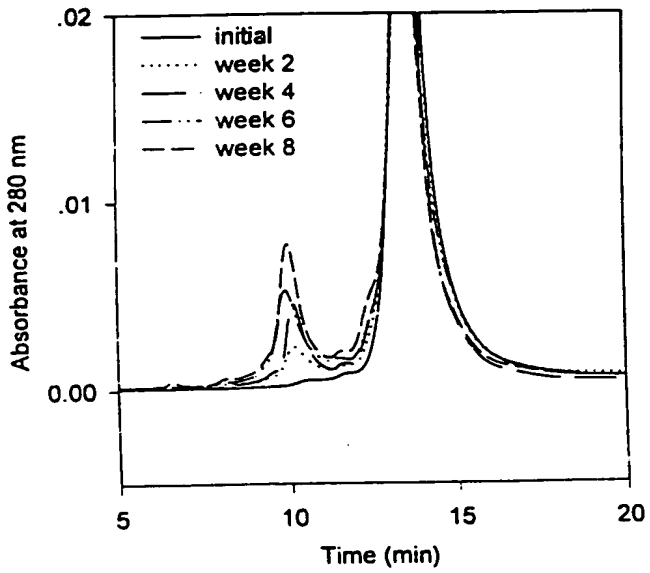
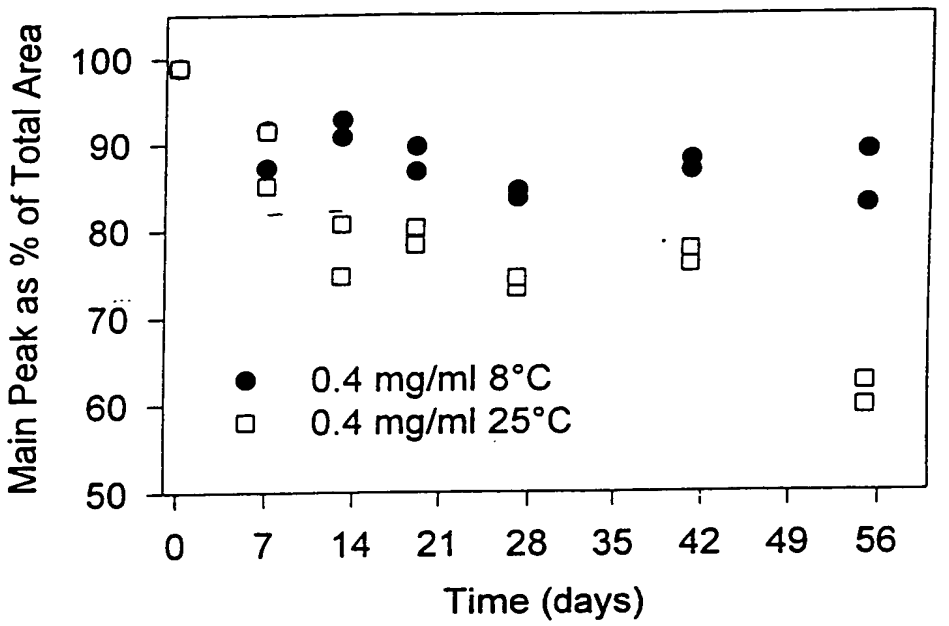


Figure 16

Stability of LIF in Acetate Buffer pH 4.0

Analysed by IEC



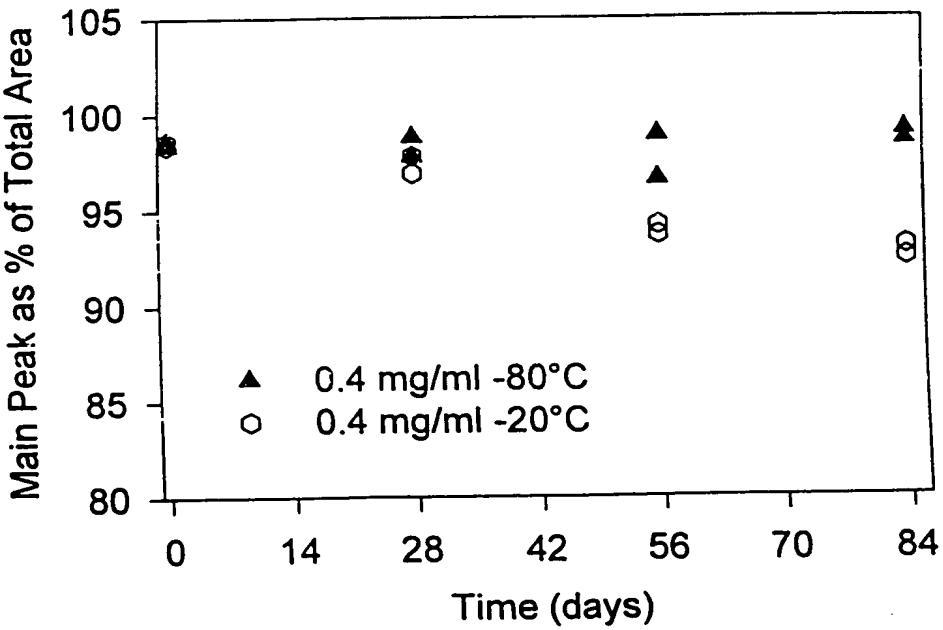
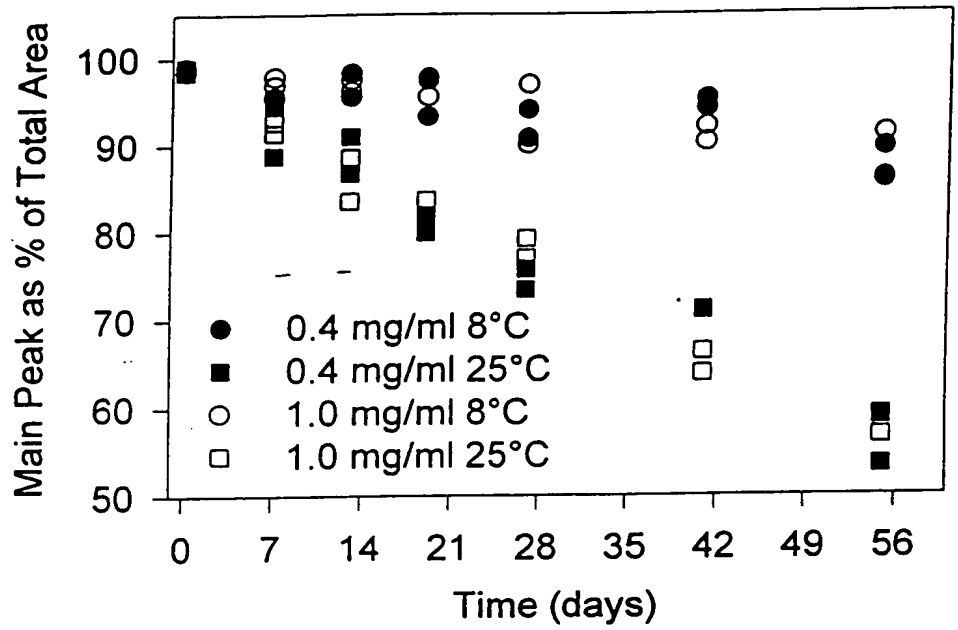


Figure 17
Stability of LIF in Acetate Buffer pH 4.5
Analysed by IEC



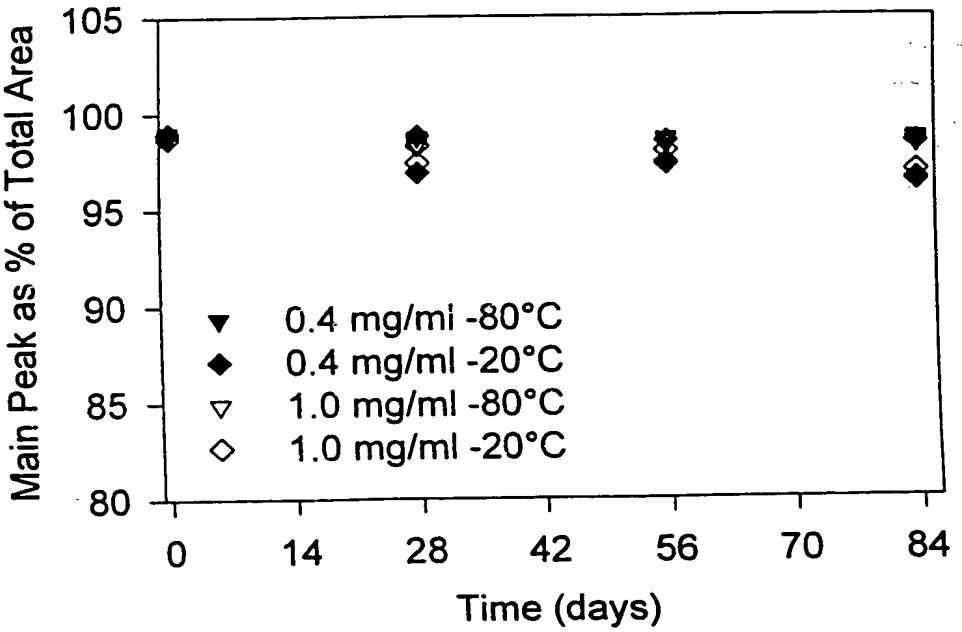
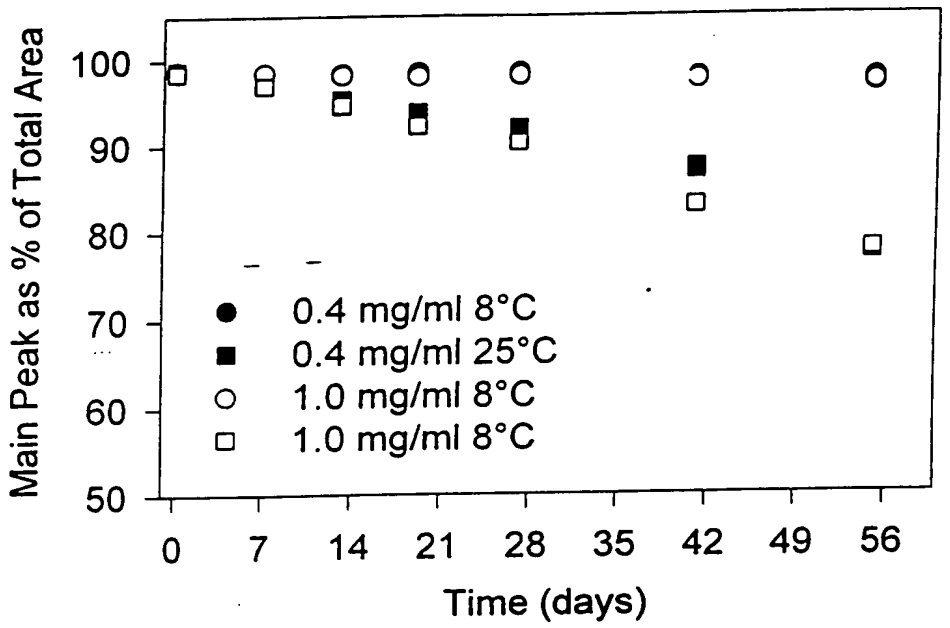


Figure 18
Stability of LIF in Citrate Buffer pH 5.0
Analysed by IEC



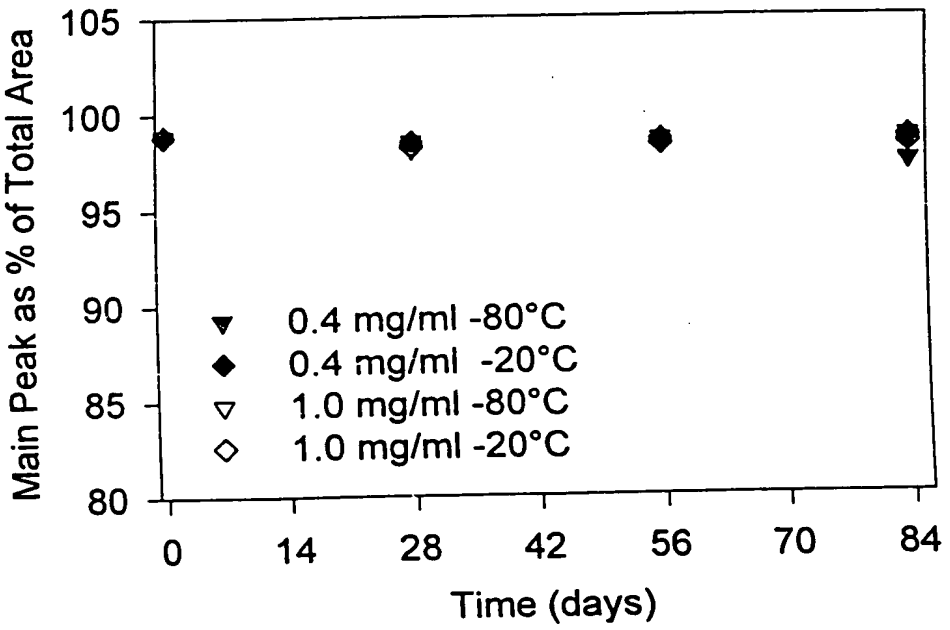


Figure 19
Concentration of LIF in Acetate Buffer
pH 4.0 Analysed by RP

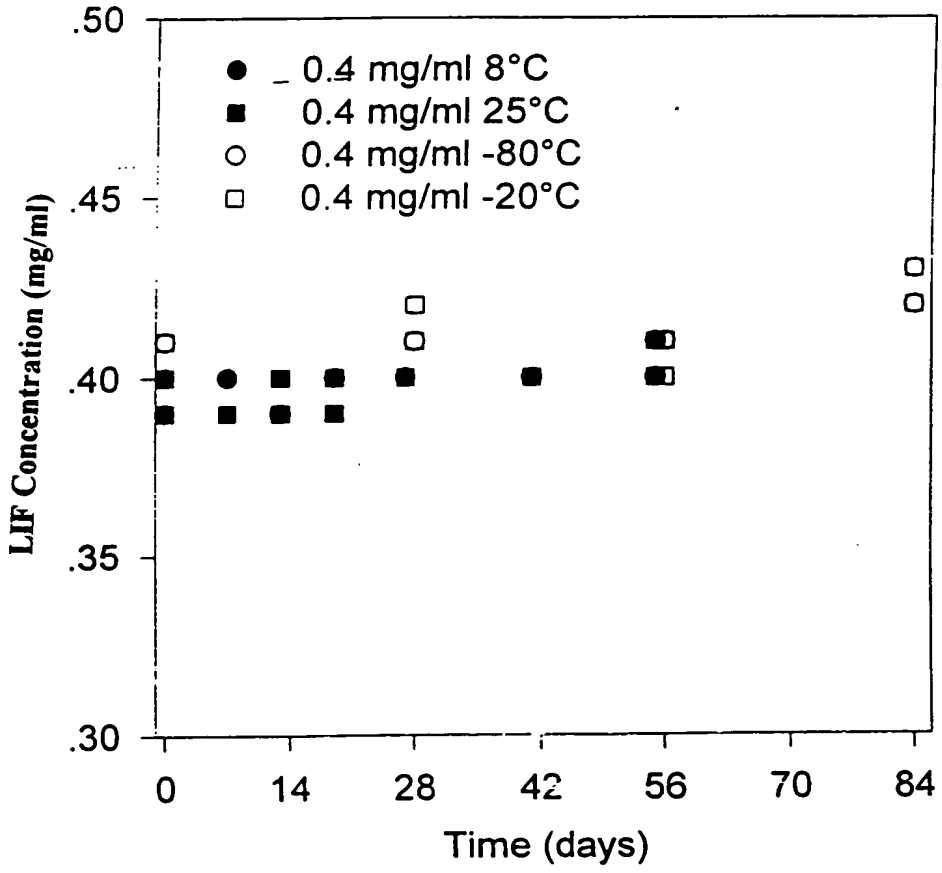
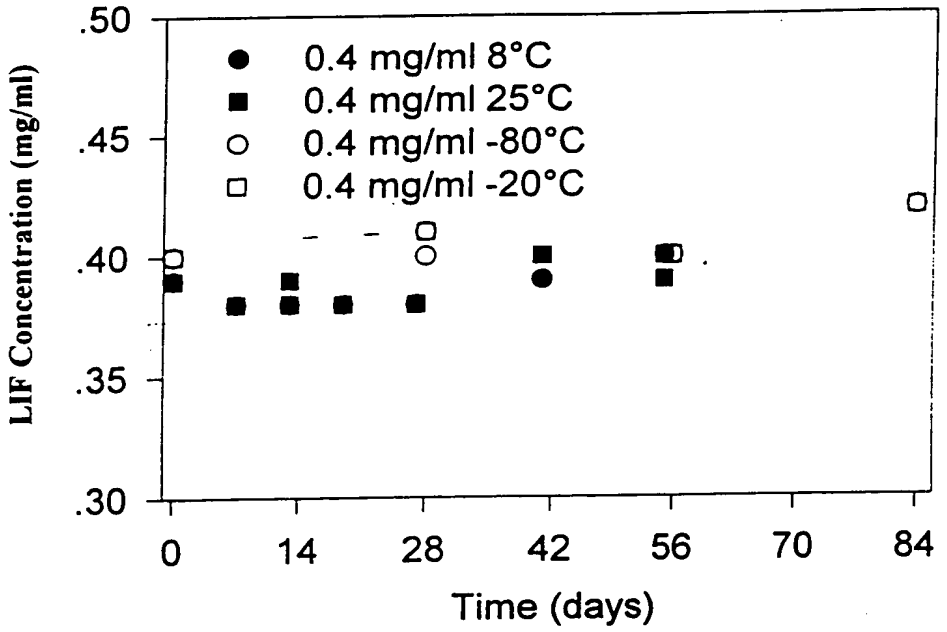


Figure 20
Concentration of LIF in Acetate Buffer
pH 4.5 Analysed by RP



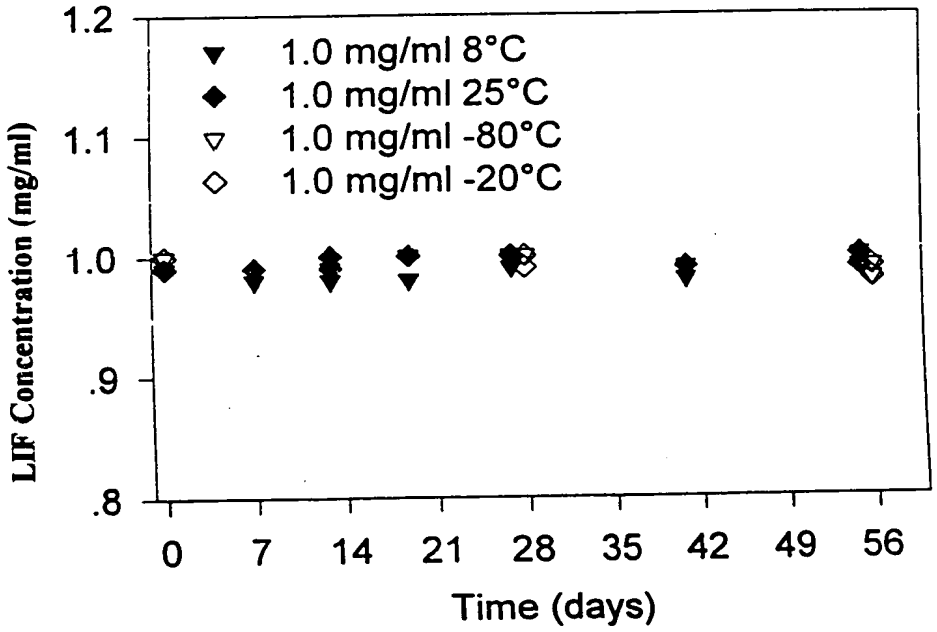


Figure 21
Concentration of LIF in Citrate Buffer
pH 5.0 Analysed by RP

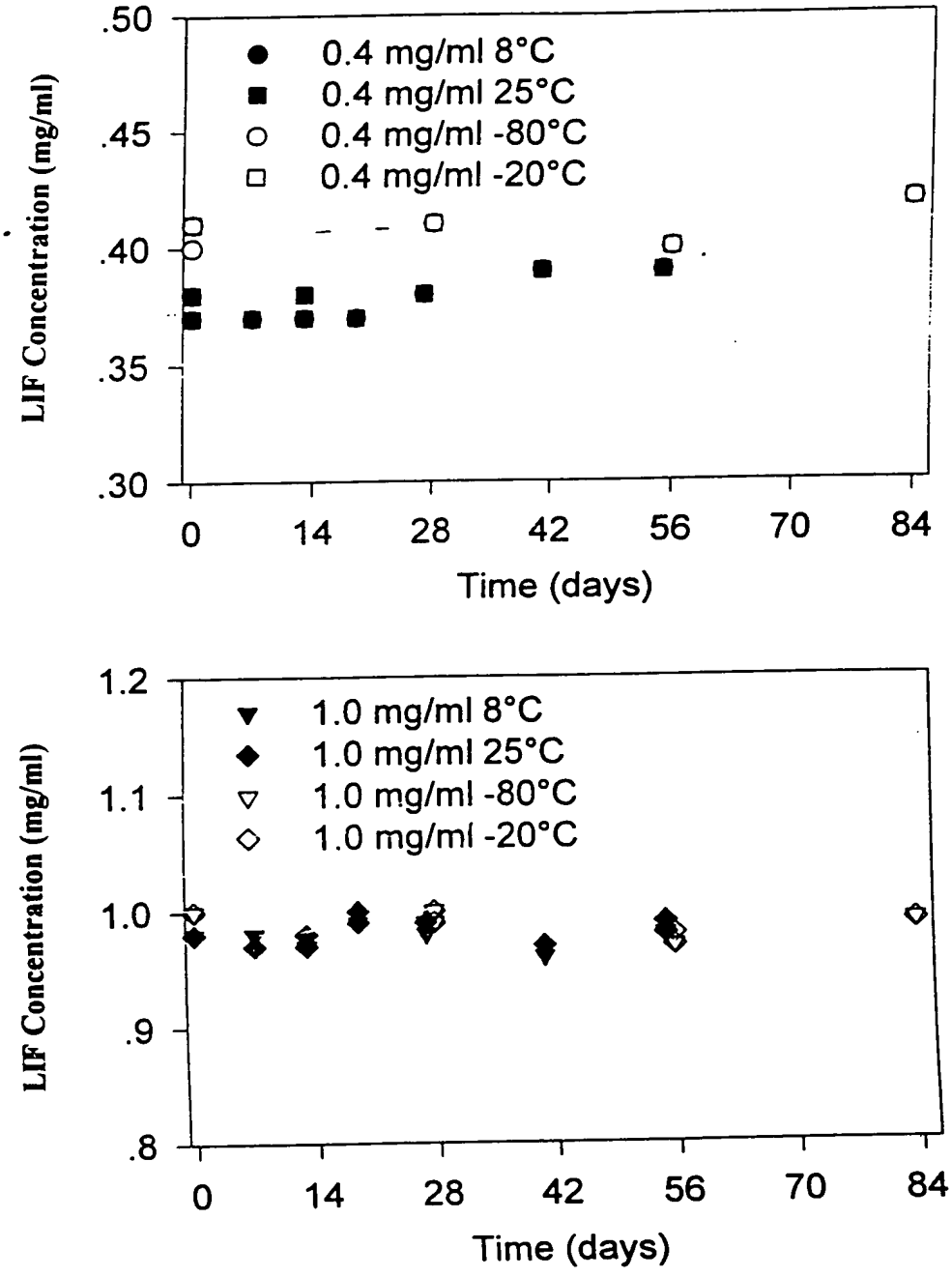


Figure 22

Concentration of LIF in Acetate Buffer pH 4.0 Analysed by SEC

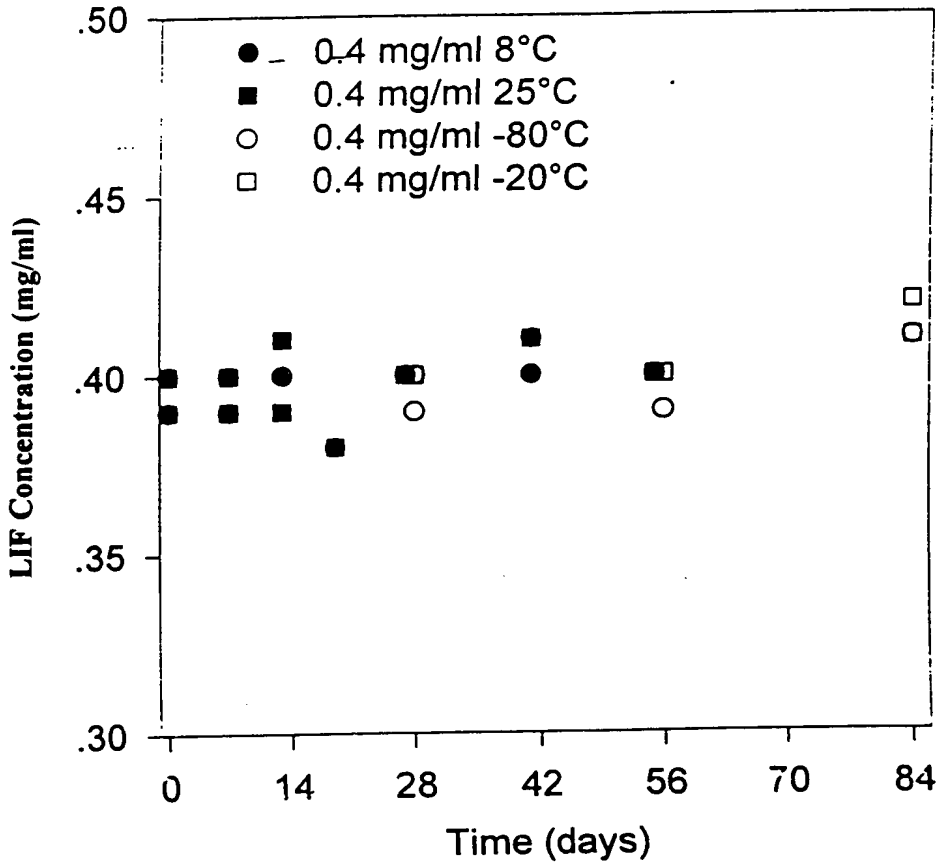


Figure 23
Concentration of LIF in Acetate Buffer
pH 4.5 Analysed by SEC

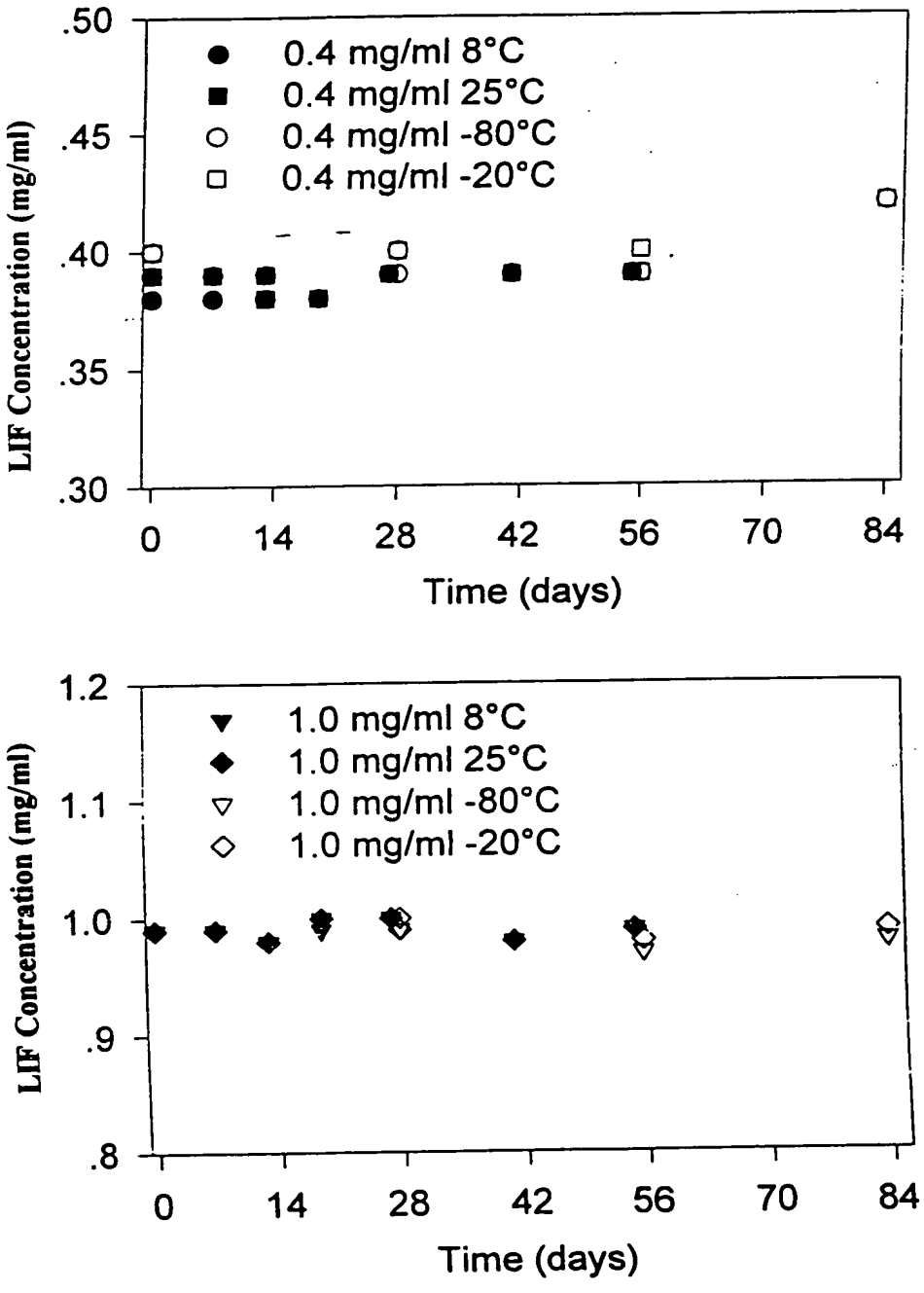
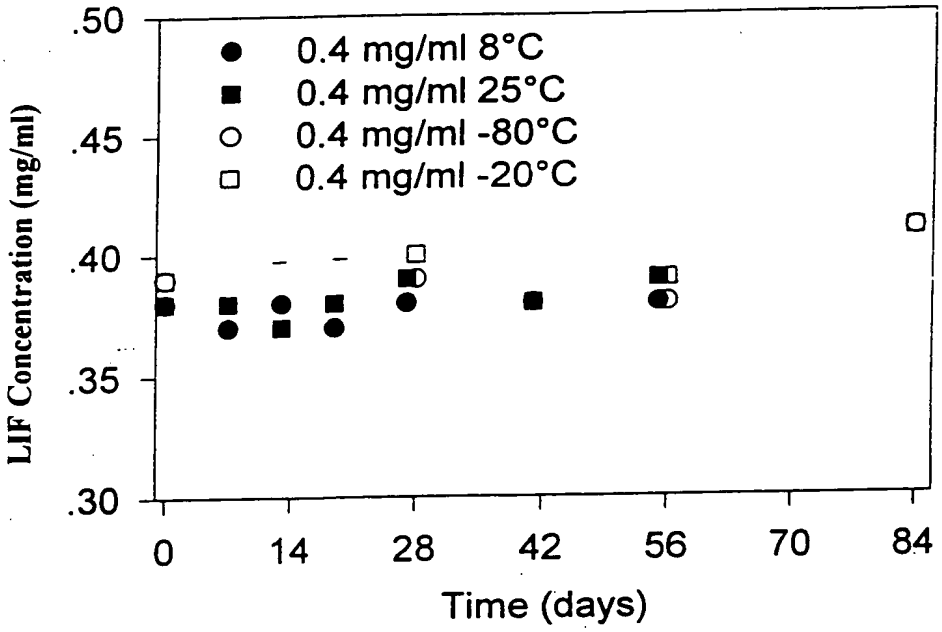


Figure 24
Concentration of LIF in Citrate Buffer
pH 5.0 Analysed by SEC



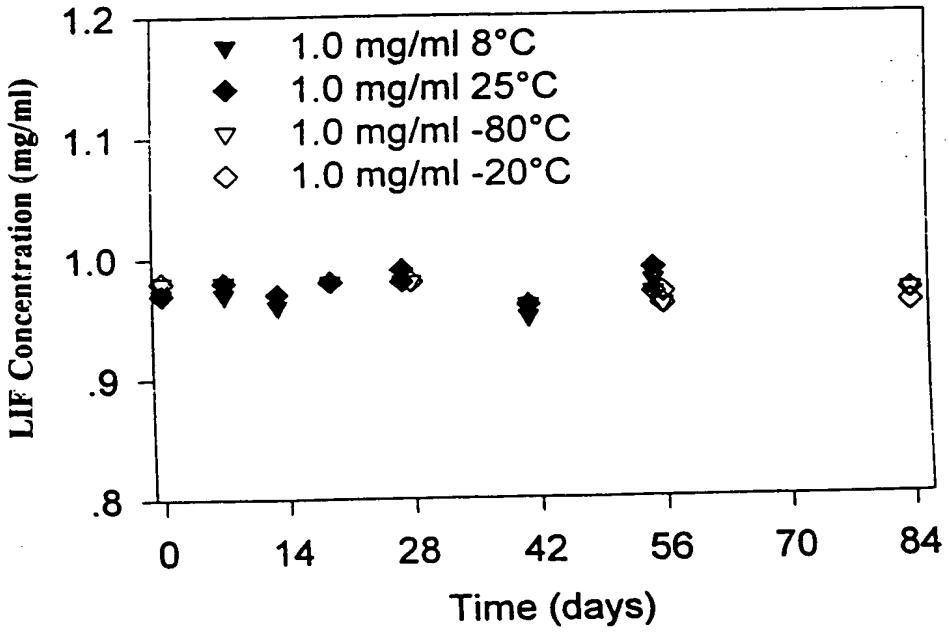


Figure 25

IEC Chromatograms for 0.4 mg/ml LIF in pH 5.5 Citrate Buffer Following Storage at 8°C

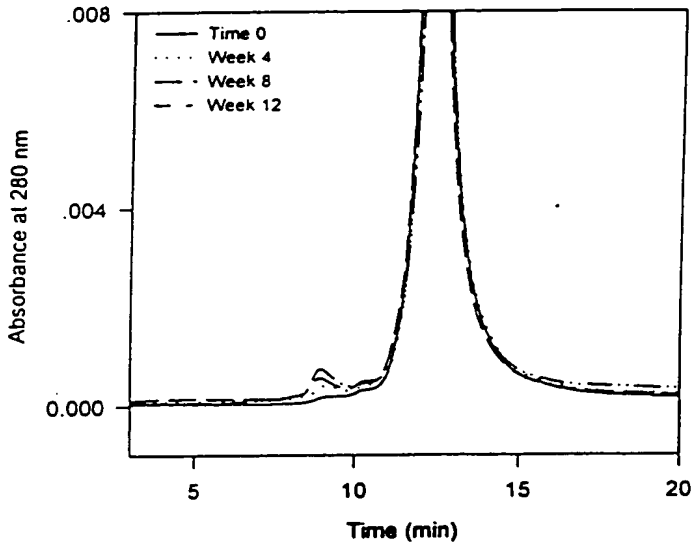


Figure 26

IEC Chromatograms for 0.4 mg/ml LIF in pH 5.5 Citrate Buffer Following Storage at 25°C

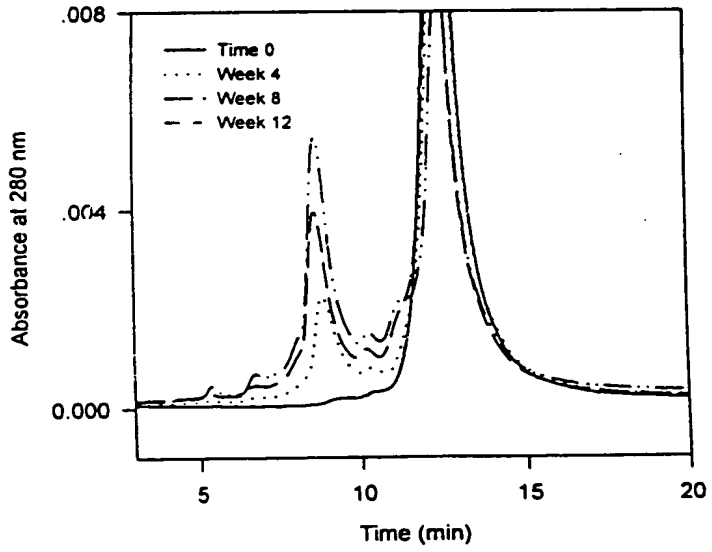


Figure 27

IEC Chromatograms for 1.0 mg/ml LIF in pH 5.5 Citrate Buffer Following Storage at 8°C

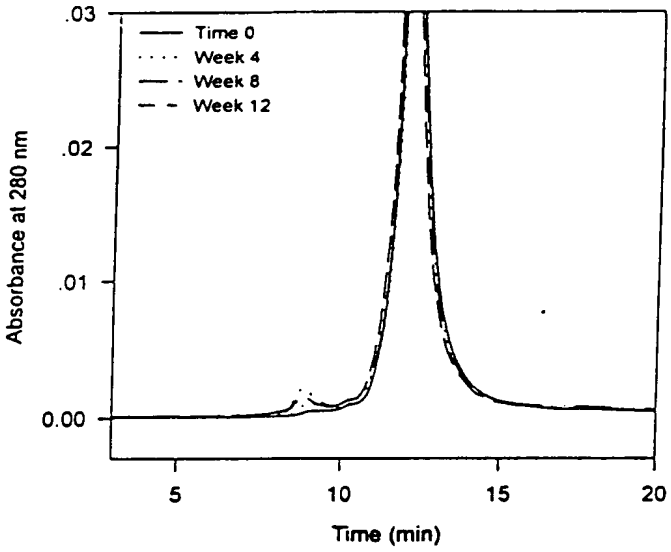


Figure 28

IEC Chromatograms for 1.0 mg/ml LIF in pH 5.5 Citrate Buffer Following Storage at 25°C

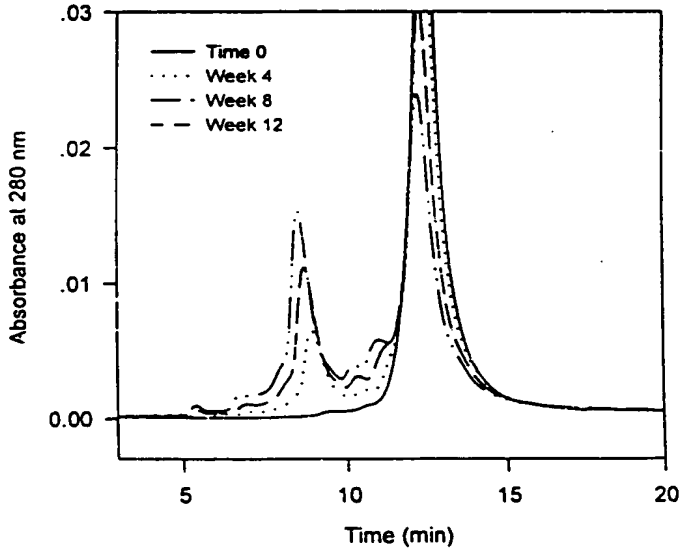


Figure 29

IEC Chromatograms for 0.4 mg/ml LIF in Citrate Buffer Following Storage for 8 weeks

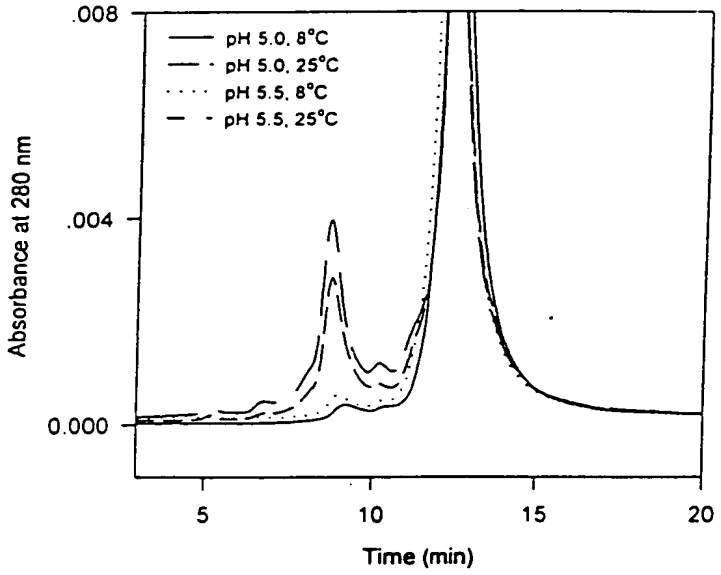


Figure 30

IEC Chromatograms for 1.0 mg/ml LIF in Citrate Buffer Following Storage for 8 weeks

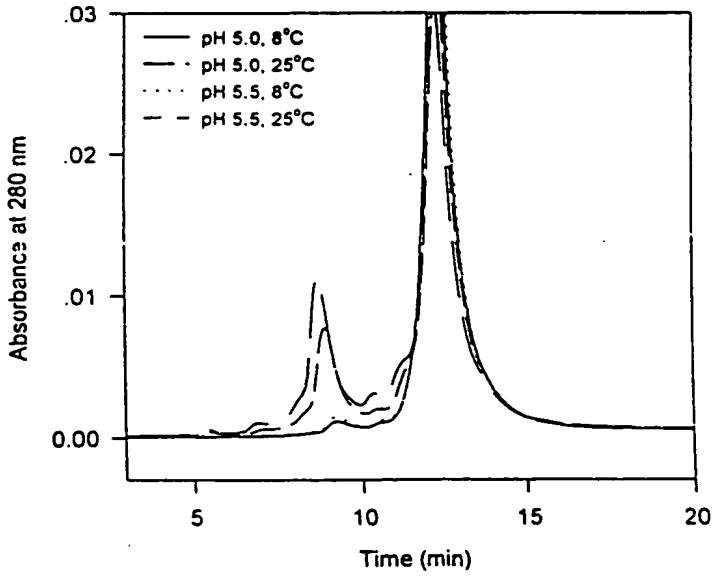


Figure 31
Stability of LIF in pH 5.5 Citrate Buffer Analysed by IEC

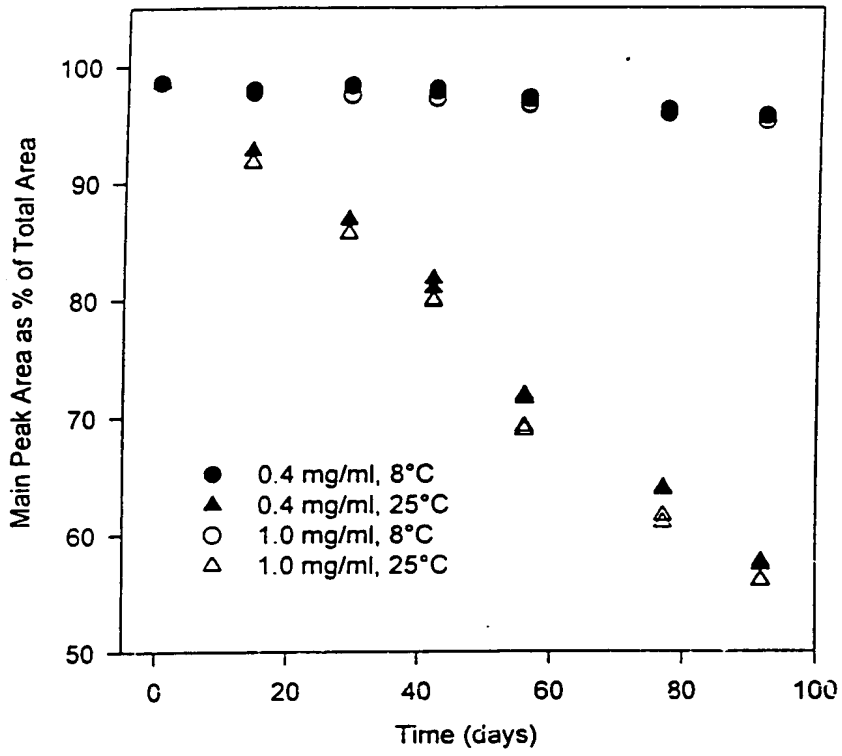


Figure 32
IEC Data Comparing 0.4 mg/ml LIF Stability at pH 5.0 and 5.5

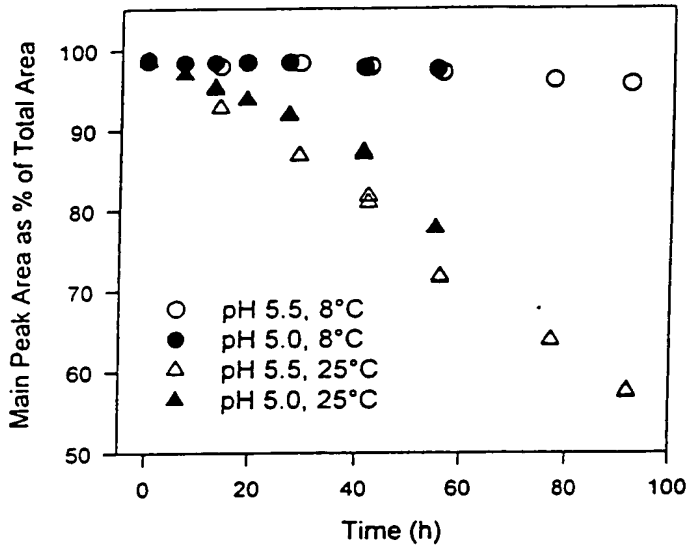


Figure 33
IEC Data Comparing 1.0 mg/ml LIF Stability at pH 5.0 and 5.5

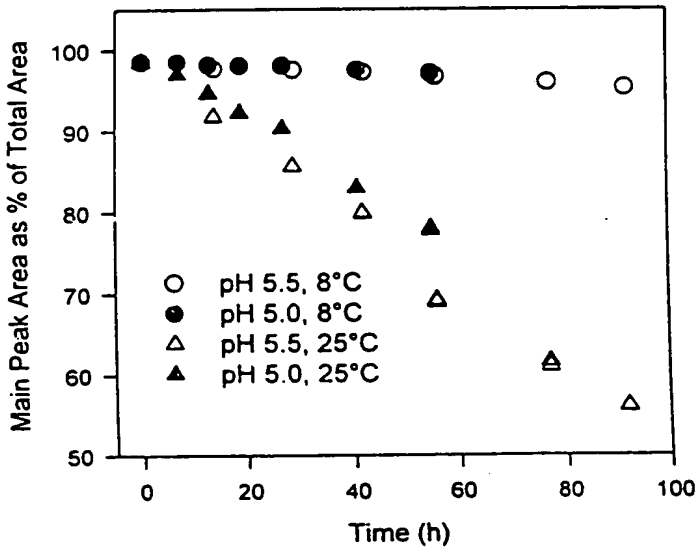


Figure 34
LIF Concentration in pH 5.5 Citrate Buffer pH 5.5 Following Storage at 8°C

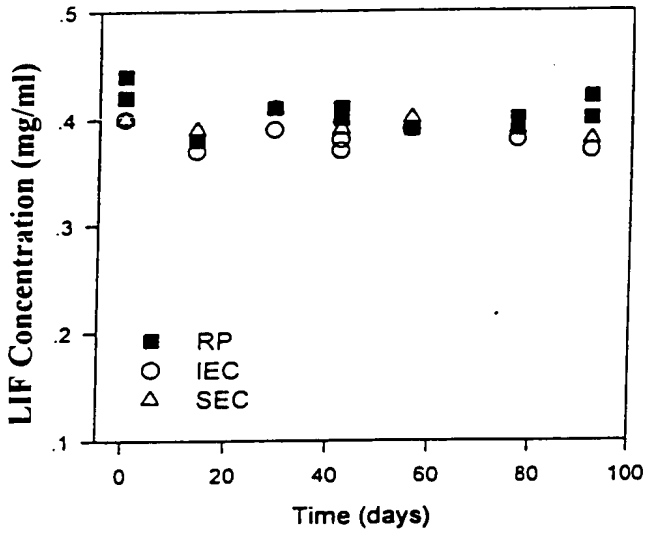


Figure 35
LIF Concentration in pH 5.5 Citrate Buffer pH 5.5 Following Storage at 25°C

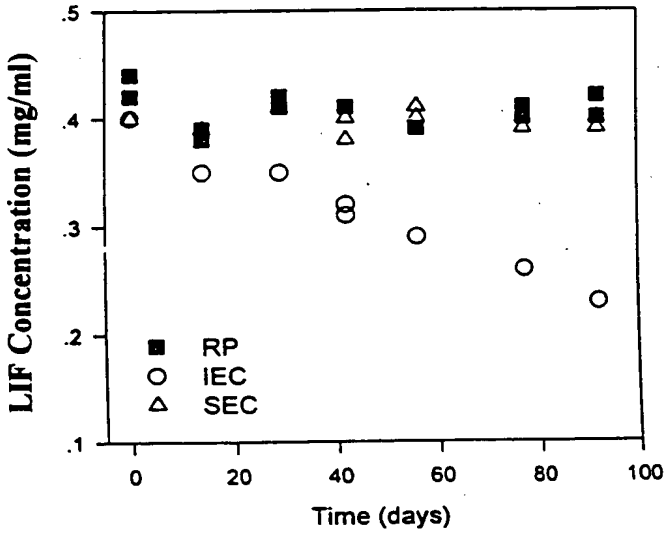


Figure 36

LIF Concentration in pH 5.5 Citrate Buffer pH 5.5 Following Storage at 8°C

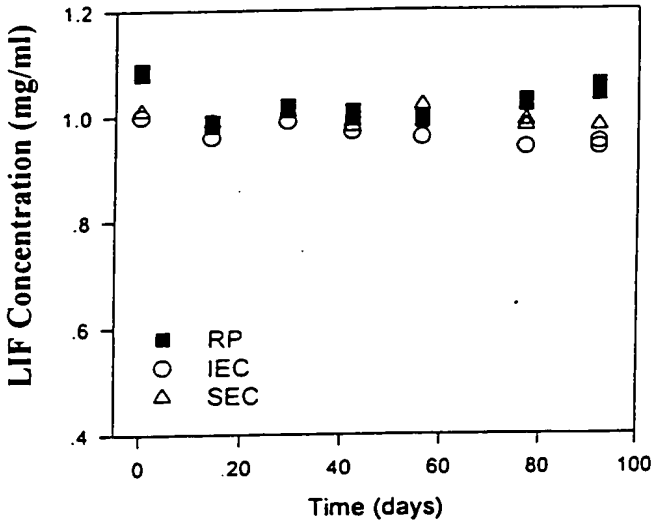


Figure 37

LIF Concentration in pH 5.5 Citrate Buffer pH 5.5 Following Storage at 25°C

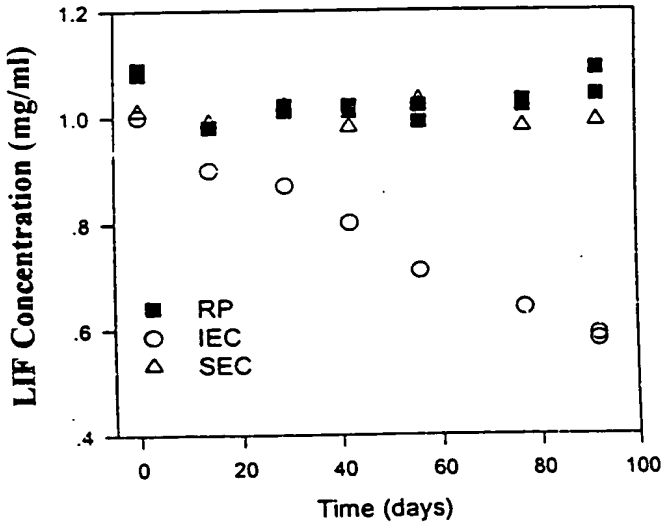


Figure 38
LIF mg/ml Stability Following Storage at 8°C Monitored by IEC

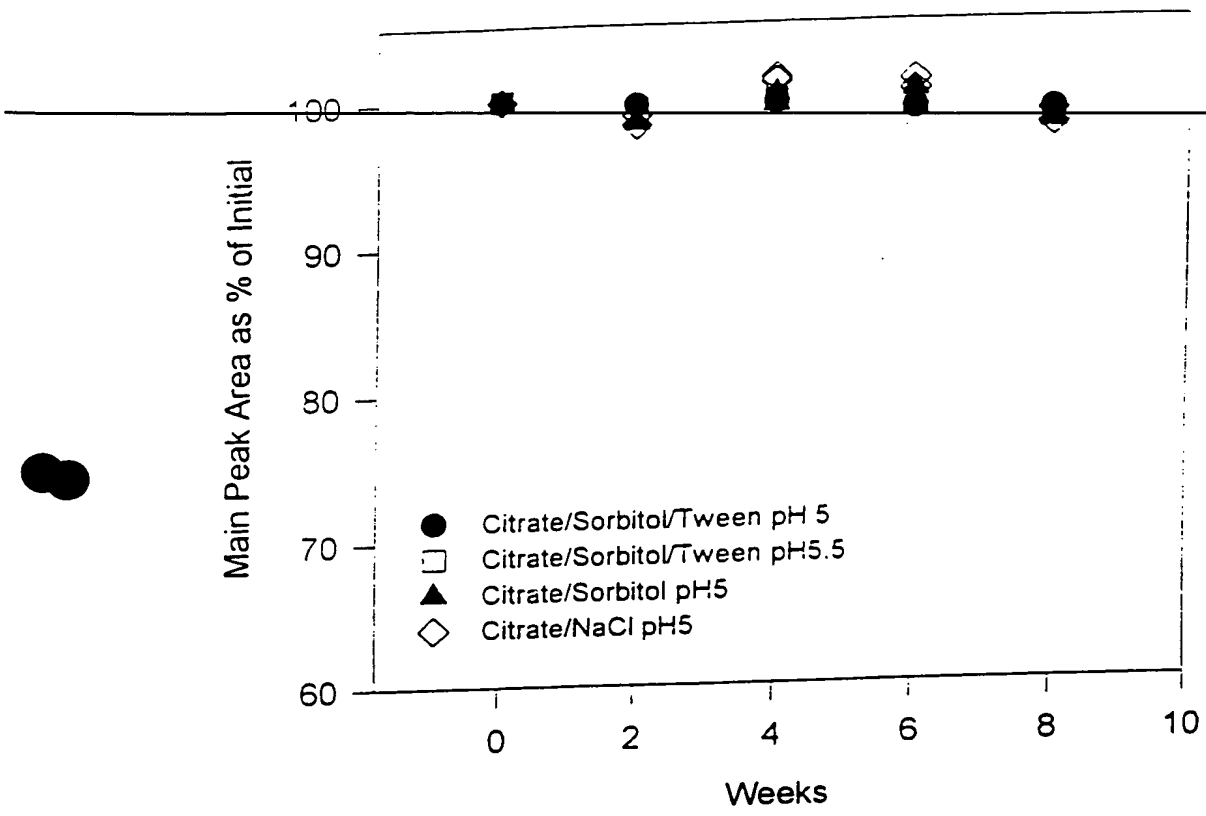


Figure 39
LIF 0.4 mg/ml Stability Following Storage at 25°C Monitored by IEC

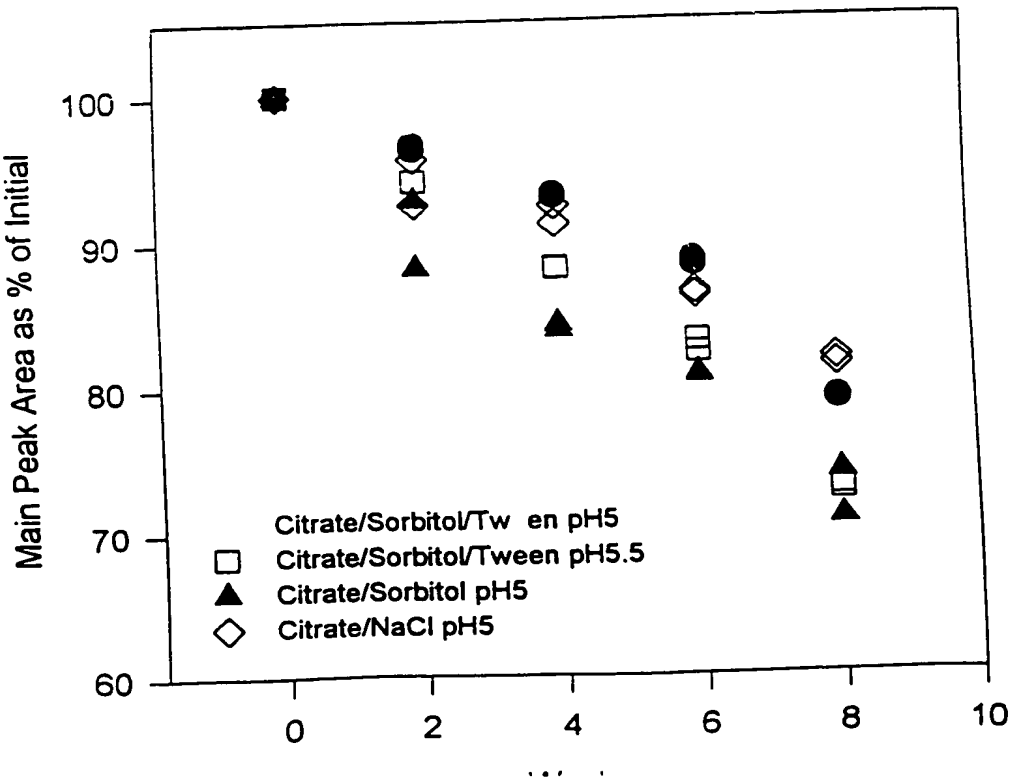


Figure 40
LIF 0.05 mg/ml - SEC Data

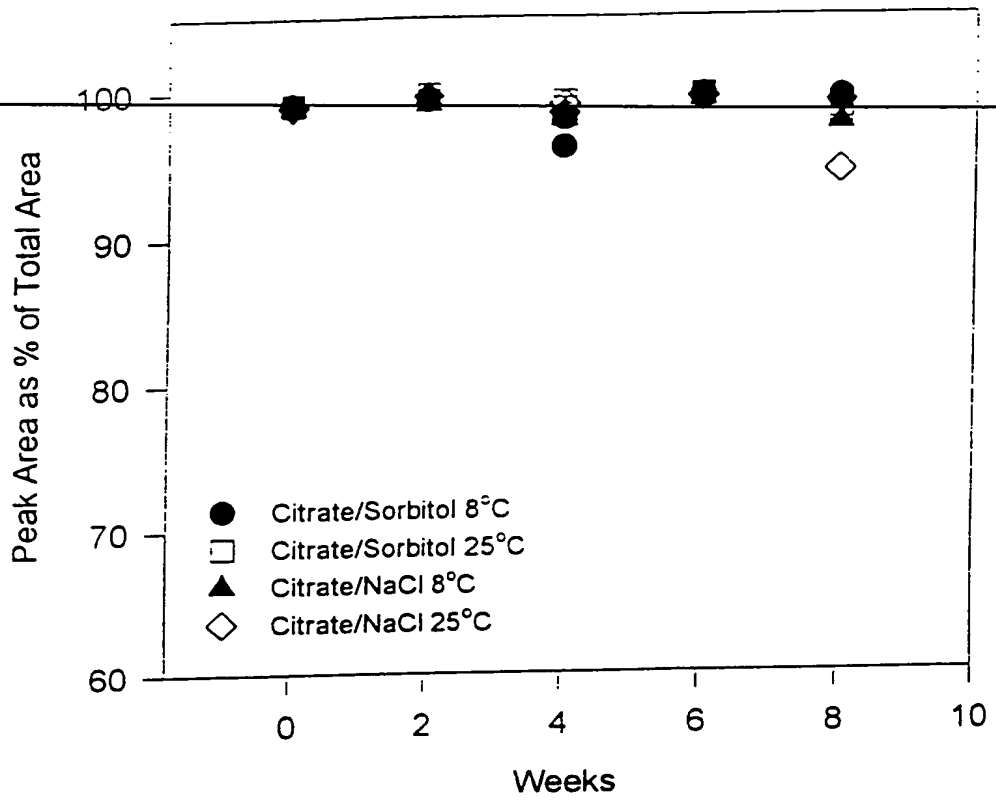


Figure 41
LIF 0.4 mg/ml - SEC Data

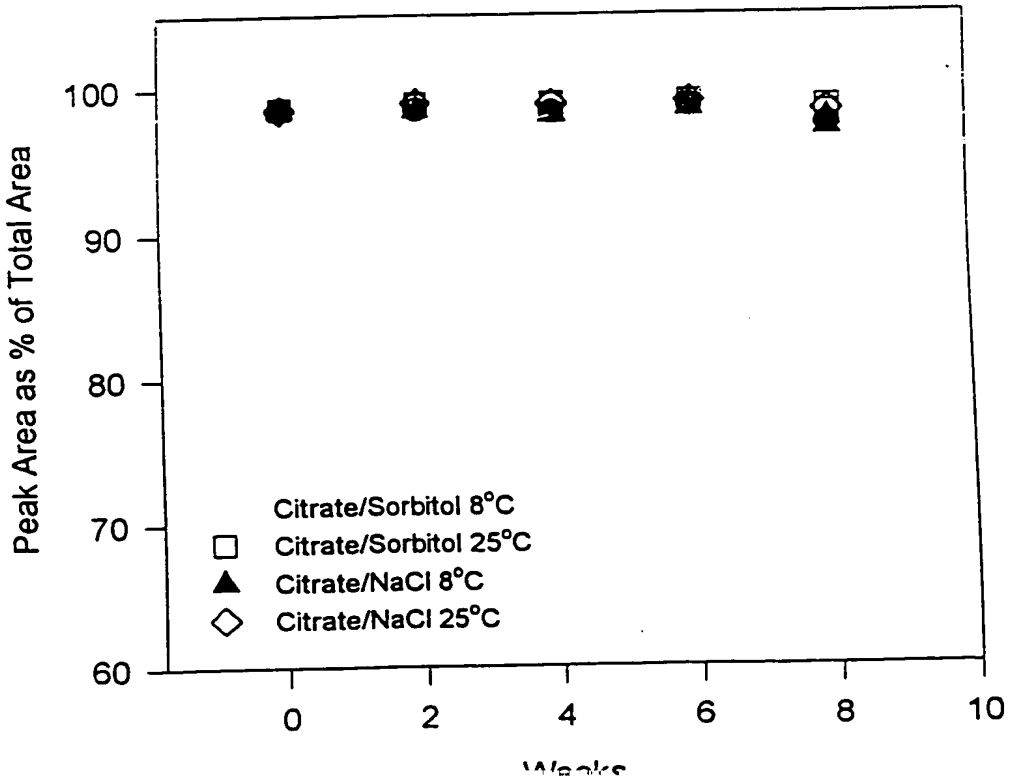


Figure 42
LIF Freeze - Thaw Cycling
Main LIF Peak by SEC

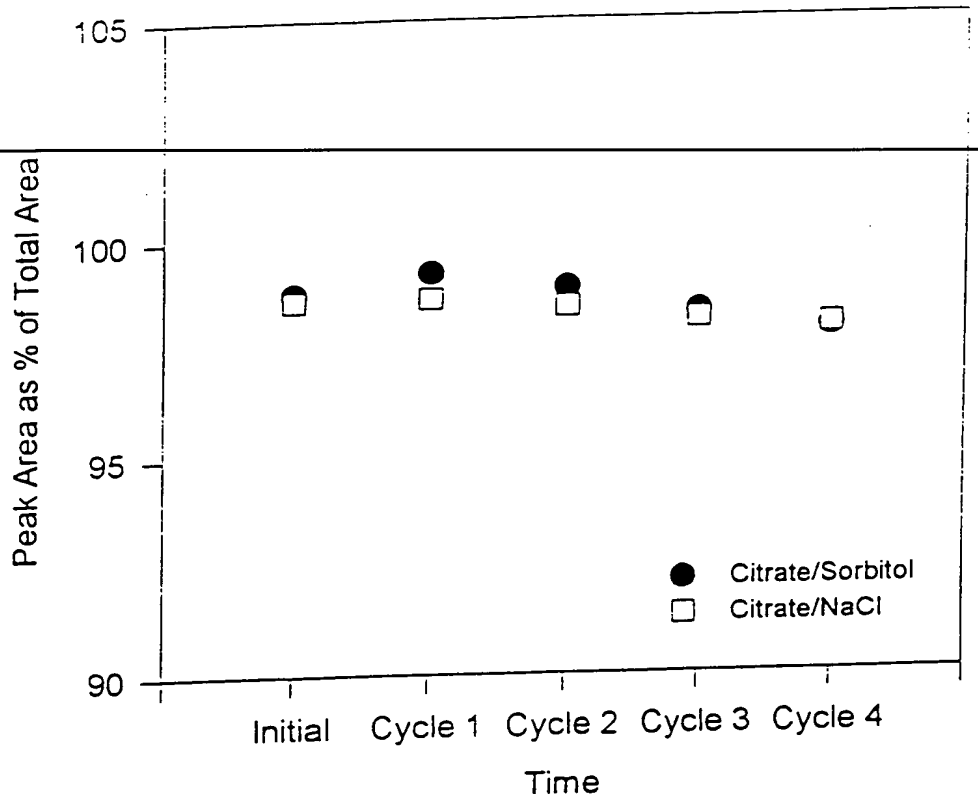


Figure 43
LIF Freeze - Thaw Cycling
Pre-Eluting Peak by SEC

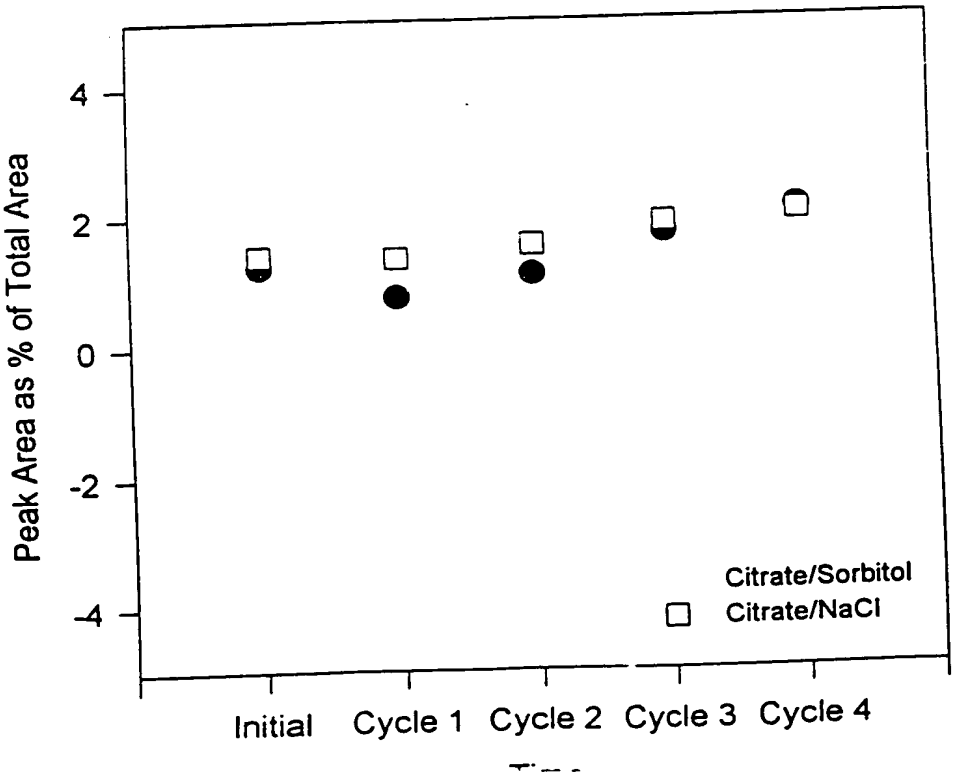


Figure 44

LIF Freeze - Thaw Cycling
by SEC